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We have shown that the expression levels of both cannabinoid receptors CB1 and CB2 are higher in human prostate cancer cells than in normal prostate epithelial cells and treatment of LNCaP cells with WIN-55,212-2 (WIN) resulted in inhibition of cell growth and induction of apoptosis. Next study was conducted to understand the mechanistic basis of these effects. Treatment of LNCaP cells with WIN resulted in i) an arrest of the cells in the G0/G1 phase of the cell cycle; ii) an induction of p53 & p27/KIP1; iii) down-regulation of cyclins decrease in the expression of cdks; iv) decrease in protein expression of pRb; v) down-regulation of E2F (1-4); and vi) decrease in the protein expression of DP1 and DP2. Similar effects were also observed when androgen-independent PC3 cells were treated with WIN(5-30 µM). We further observed sustained up regulation of ERK1/2, and inhibition of PI3k/Akt pathways in WIN-55,212-2 treated cells. Inhibition of ERK1/2 abrogated WIN indued cell death suggesting that sustained activation of ERK1/2 leads to cell-cycle dysregulation and arrest of cells in G0/G1 phase subsequently leading to an induction of apoptosis. Further, WIN treatment of cells resulted in a dose-dependent increase in Bax/Bcl-2 ratio in such a way that favors apoptosis. The induction of apoptosis proceeded through down regulation of caspases 3, 6, 7, and 9 and cleavage of PARP. To establish in vivo relevance of these in vitro findings, we implanted athymic nude mice with androgen-responsive CWR22R•1 cells which form rapid tumors and secrete PSA in the blood stream of the host. As compared to untreated animals, WIN treated mice (0.5 mg/kg b.wt, i.p, alternate day) exhibited significant inhibition in the tumor growth with significant reduction in PSA secretion in the serum. In animals without WIN treatment, targeted tumor volume of 1200 mm3 was reached at 35 days post-tumor inoculation; whereas this tumor volume was attained in 51 days in WIN treated. Based on these results we suggest that cannabinoids could used in the management of prostate cancer.

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Background

Because prostate cancer has become the most common cancer diagnosed in men, developing novel targets and mechanism based agents for its treatment has become a challenging issue. In recent years, cannabinoids, the active components of *Cannabis sativa linnaeus* (marijuana) and their derivatives are drawing renewed attention because of their diverse pharmacological activities such as cell growth inhibition, anti-inflammatory effects and tumor regression (1-5). Cannabinoids have shown to induce apoptosis in gliomas (6), PC-12 pheochromocytoma (7), CHP 100 neuroblastoma (8) and hippocampal neurons (9) in vitro, and most interestingly, regression of C6-cell gliomas in vivo (10). Further interest in cannabinoid research came from the discovery of specific cannabinoid system and the cloning of specific cannabinoid receptors (10). These diversified effects of cannabinoids are now known to be mediated by the activation of specific G protein-coupled receptors that are normally bound by a family of endogenous ligands, the endocannabinoids (11, 12). Two different cannabinoid receptors have been characterized and cloned from mammalian tissues: the "central" CB_I receptor (13), and the "peripheral" CB_2 receptor (14). We have shown that the expression levels of both cannabinoid receptors CB_1 and CB_2 are higher in human prostate cancer cells than in normal prostate epithelial cells and treatment of LNCaP cells with WIN-55,212-2 (a mixed CB_1/CB_2 agonist) resulted in inhibition of cell growth and induction of apoptosis (15). Next study was conducted to understand the mechanistic basis of these effects. Treatment of LNCaP cells with WIN-55,212-2 (1-10 µM; 24 h) resulted in i) an arrest of the cells in the G_0/G_1 phase of the cell cycle; ii) an induction of p53 & p27/KIP1; iii) down-regulation of cyclins D1, D2, E; and iii) decrease in the expression of cdk -2, -4, and -6; iv) decrease in protein expression of pRb; v) down-regulation of E2F (1-4); and vi) decrease in the protein expression of DP1 and DP2. Similar effects were also observed when androgen-independent PC3 cells were treated with WIN-55,212-2 (5-30 µM). We further observed sustained up regulation of ERK1/2, and inhibition of PI3k/Akt pathways in WIN-55,212-2 treated cells. Inhibition of ERK1/2 abrogated WIN-55,212-2-indued cell death suggesting that sustained activation of ERK1/2 leads to cell-cycle dysregulation and arrest of cells in G_0/G_1 phase subsequently leading to an induction of apoptosis. Further, WIN-55,212-2 treatment of cells resulted in a dose-dependent increase in Bax/Bcl-2 ratio in such a way that favors apoptosis. The induction of apoptosis proceeded through down regulation of caspases 3, 6, 7, and 9 and cleavage of PARP. To establish *in vivo* relevance of these *in vitro* findings, we implanted athymic nude mice with androgen-responsive CWR22Rv1 cells which form rapid tumors and secrete PSA in the blood stream of the host. As compared to untreated animals, WIN treated mice (0.5 mg/kg b.wt, i.p, alternate day) exhibited significant inhibition in the tumor growth with significant reduction in PSA secretion in the serum. In animals without WIN treatment, targeted tumor volume of 1200 mm^3 was reached at 35 days post-tumor inoculation; whereas this tumor volume was attained in 51 days in WIN treated mice. Since angiogenesis is an essential component to primary tumor growth and metastasis, we next assessed the effect of WIN treatment on the markers of cell proliferation, angiogenesis and metastasis. Protein expression of PCNA, a marker of cell proliferation was considerably lower (45%) in tumors of WIN treated mice as compared to untreated animals. Protein expression of angiopoetins and VEGF, members of the

vascular endothelial growth factor family that participate in the formation of blood vessels were also evaluated. Tumor tissues from WIN treated mice had notably lower expression of both angiopoetin-1 (41%) and angiopoetin-2 (38%) and showed marked decrease (47%) in the expression of VEGF positive cells. In the next series of experiments we determined the effect of WIN on the expression of proteins involved in metastasis. The balance between matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP) is an essential factor in the aggressiveness of several cancers. We observed that MMP to TIMP ratio in WIN treated mice was tilted towards TIMP expression suggesting inhibition of MMP expression.

Based on these data we suggest that cannabinoid receptor agonists should be considered as novel agents for the management of prostate cancer.

The major purpose of this research supported by the Award W81XWH-04-1-0217 is to establish whether cannabinoid receptors could prove to be useful targets for the treatment of prostate cancer.

Body

Specific Aims: The following specific aims were proposed

1. To investigate the consequences of the activation of cannabinoid receptors in human prostate cancer cells *in vitro*.
(a) To investigate whether the activation of cannabinoid receptors impart inhibitory effect on cell growth/cell viability in human prostate cancer cells without affecting normal cells.
(b) To investigate whether cannabinoids selectively induces apoptosis in human prostate carcinoma cells without affecting normal cells.
(c) To investigate whether cannabinoids is associated with inhibition of angiogenesis and PSA levels in human prostate carcinoma cells.

2 To investigate the consequences of the activation of cannabinoid receptors under *in vivo* situation, in athymic nude mice implanted with human prostate cancer cells.
Specific aim II of the proposal was designed to investigate the therapeutic potential of cannabinoids under *in vivo* situation on cell proliferation, apoptosis, markers of angiogenesis and PSA levels. We proposed to employ athymic nude mice and LNCaP cells for this part of the work. In the experimental plan, we proposed to treat the animals in two different ways: i) the animals will be given cannabinoid treatment (WIN-55,212-2, i.p. 0.5 mg b.wt) simultaneously with the implantation of $1x10^6$ LNCaP cells, and ii) the animals will be given cannabinoids treatment (WIN-55,212-2, i.p. 0.5 mg b.wt) after the development of a sizable tumor. As the experiment progressed tumor growth was found to be slower than expected. From these experiments we learned that number of cells we have used may not be optimal for tumor growth which could mask the effects and or

LNCaP cells may not be appropriate for these studies. We implanted athymic nude mice with androgen-responsive CWR22Rv1 cells which form rapid tumors and secrete PSA in the blood stream of the host. As compared to untreated animals, WIN treated mice (0.5 mg/kg b.wt, i.p, alternate day) exhibited significant inhibition in the tumor growth with significant reduction in PSA secretion in the serum.

Experimental Details

Materials. R-(+)-WIN 55,212-2 (2, 3 Dihydro-5-methyl -3 ([morpholinyl]methyl) pyrollo (1,2,3 de)-1,4-benzoxazinyl]- [1-napthaleny] methanone, $C_{27}H_{26}N_2O_3.CH_3SO_3H$ was purchased from Sigma (St. Louis, MO). CB_1 receptor antagonist SR141716 (SR1) and CB_2 receptor antagonist SR144528 (SR2) were procured from Dr. Herbert H. Seltzman (National Institute on Drug Abuse, Division of Neuroscience and Behavioral Research, through RTI International, Research Triangle Park, NC). Dulbecco's modified Eagle's medium and Fetal Bovine Serum (FBS) were procured from GIBCO, Invitrogen Corporation (Grand Island, N.Y). Human PSA ELISA kit from Yes Biotech laboratories (Ontario, Canada) and annexin-V-FLUOS staining kit from Roche Diagnostic Corporation, Indianapolis, IN, were procured. Antibiotic (Penicillin and Streptomycin) used were obtained from Cellgro Mediatech, Inc. (Herndon, VA). Apo-Direct kit for measuring apoptosis by flow cytometry was procured from Apo-Direct San Diego, CA. RNA isolation kit (Qiagen, Inc. Valencia, CA). ERK1/2 inhibitor PD98059 was purchased from Tocris Biosciences (Ellisville, MO). Monoclonal antibodies for PSA, AR, PCNA and VEGF, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies for CB_1 and CB_2 were purchased from Cayman Chemical Company (Ann Arbor, Michigan). The mono- and polyclonal antibodies (p53, cdk2, 4 and 6, KIP1/p27, E2F-3 and DP-2) were obtained from Santa Cruz Biotechnology Inc. (CA). The human reactive monoclonal and polyclonal antibodies (cyclins D1, D2, E, pRb, E2F-1, E2F-2, E2F-4 and DP-1) were obtained from Labvision (Fremont, CA). Monoclonal and polyclonal antibodies for anti-PARP, Bcl-2 Bax were purchased from Upstate Biotechnology (Lake Placid, NY). Anti- PARP (116KD) and anti-PI3K kinase (p85) was purchased from Upstate Biotechnology (Lake Placid, NY) and anti- PARP (p85) was purchased from Promega (Madison, WI). Anti-phospho-ERK1/2 p42/44 was purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit secondary horseradish peroxidase conjugate was obtained from Amersham Biosciences Limited (Buckinghamshire, England). Protein was estimated using BCA Protein assay kit obtained from Pierece (Rockford, IL).

Cell Culture

The LNCaP, DU145, PC-3, CWR22Rv1, CA-HPV-10 and PZ-HPV-7 cells were obtained from ATCC (Manassas, VA,). LNCaP and DU145 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. PC-3 and CWR22Rv1cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. CA-HPV-10 and PZ-HPV-7

were grown in keratinocyte-serum free medium (GIBCO-BRL 17005-042) supplemented with 5 ng/mL human recombinant EGF and 50 µg/mL bovine pituitary extract. Human prostate epithelial cells (PrEC) were obtained from Cambrex Bioscience (Walkersville, MD) and grown in prostate epithelial basal cell medium (Cambrex Bioscience, Walkersville, MD) as per the vendors instructions. The cells were maintained under standard cell culture conditions at 37 ^{0}C and 5% CO_2 in a humid environment.

Treatment of Cells

WIN-55,212-2, (dissolved in DMSO) was used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies cells were treated with WIN-55,212-2 at 1.0, 2.5, 5.0, 7.5, 10.0 µM final concentrations for 24 h in complete cell medium, whereas for time-dependent studies the cells (50-60% confluent) were treated with 5 µM dose of WIN-55,212-2 for 24, 48 and 72 h. For time-dependent study, we included a control treated with DMSO for 72 h because it was the longest time-point post WIN-55,212-2 treatment in our experimental protocol. To establish the role of CB_1 and CB_2 receptors in WIN-55,212-2 induced inhibitory effects two experiments were performed. In the first experiment, cells were treated with 2 µM of SR141716 or SR144528 alone for 24 h. In the second experiment cells pretreated with each of these antagonists (2µM) for 3 h followed by incubation with 7.5 µM WIN-55,212-2 for 24 h. In pilot experiments, it was established that DMSO (0.1% v/v) had no effects when measured at 24, 48 or 72 h.   WIN-55,212-2, was used for the treatment of CWR22Rv1 cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies cells were treated with WIN-55,212-2 at 5.0, 10, 15, 20, 25, 30 µM final concentrations for 24h in complete cell medium. Cells treated with vehicle alone were used as control.

Cell Viability by MTT assay

The effect of WIN-55,212-2 on the viability of cells was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. The cells were plated at 1×10^4 cells per well in 200 µl of complete culture medium containing 1.0, 2.5, 5.0, 7.5 and 10.0 µM concentrations of WIN-55,212-2 in 96-well microtiter plates for 24 and 48 h at 37 ^{0}C in a humidified chamber. Each concentration of WIN-55,212-2 was repeated in 10 wells. After incubation for specified times at 37°C in a humidified incubator, MTT reagent (4 µL, 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4°C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 µL). Absorbance was recorded on a microplate reader at 540 nm wavelength. The effect of WIN-55,212-2 on growth inhibition was assessed as percentage inhibition in cell growth where vehicle-treated cells were taken as 100% viable.

Enzyme Linked Immunoabsorbent Assay for PSA

The human PSA ELISA kit was used for the quantitative determination of PSA levels in culture medium according to the vendor's protocol. This kit uses a technique of quantitative sandwich immunoassay for determination of PSA with an estimated sensitivity of 1ng/mL PSA antigen.

Detection of Apoptosis and Necrosis by Confocal Microscopy

The Annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells according to vendor's protocol. This kit uses a dual-staining protocol in which the apoptotic are stained with annexin V (green fluorescence), and the necrotic cells are stained with propidium iodide (PI; red fluorescence). LNCaP cells were grown to about 70% confluence and then treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, 10.0 μM) for 24 h. The fluorescence was measured by a Zeiss 410 confocal microscope (Thornwood, NY). Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514–540 nm bandpass barrier filter. Images of red PI fluorescence were collected using a 568 nm excitation light from the argon/krypton laser, a 560 nm dichroic mirror, and a 590 nm long pass filter. In a selected field, the cells stained with annexin V and PI as well as unstained cells were counted to ascertain the extent of apoptosis and necrosis.

Quantification of Apoptosis by Flow Cytometry

The cells were grown at density of 1×10^6 cells in 100 mm culture dishes and were treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, 10.0 μM doses) for 24 h. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by use of an Apo-direct apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) as per manufacturer's protocol. The labeled cells were analyzed by flow cytometry.

Quantitative Real-Time PCR for mRNA expression of AR and PSA

Total RNA was isolated from LNCaP cells using RNAeasy kit according to vendor's protocol. The ratio of optical densities of RNA samples at 260 nm and 280 nm was consistently > 1.8. cDNA was synthesized by reverse transcription of 1 μg of extracted RNA with 200 U MMLV reverse transcriptase (Promega, Madison, WI) in the presence of oligo dT and dNTP (Promega, Madison, WI). AR and PSA were amplified using a Failsafe™ Real-Time PCR system obtained from Epicentre, Madison, WI. Thermal cycler used for amplification was ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers were designed as follows: AR Forward, 5'-AAGACGCTTCTACCAGCTCACCAA; reverse, 5'-TCCCAGAA-AGGATCTTGGGCACTT; PSA Forward, 5'-ACTCACAGCAAGGATGGAGCTGAA; reverse, 5'-TGAGGGTTGTCTGGAGGACTTCAA. Cycler was programmed at the following conditions (a) initial denature at 94 ^{0}C for 2 min, followed by 35 cycles of (b)

94 ^{0}C for 40 sec (c) anneal the primer template at 58 ^{0}C for 40 sec d) extend at 72 ^{0}C at 40 sec.

Quantification of Apoptosis & Cell Cycle Analysis by Flow Cytometry

The cells were grown at density of 1 X 10^6 cells in 100 mm culture dishes and were treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, 10.0 µM doses) for 24 h. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by use of an Apo-direct apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) as per manufacturer's protocol. The labeled cells were analyzed using a FACScan benchtop cytometer (BD Biosciences, San Jose, CA) at the UWCCC Flow Cytometry Facility in the University of Wisconsin. Results were analysed using ModFit LT software (Verity Software House, Topsham, ME) for cell cycle and WinMD1 version 2.8 software for quantification of apoptosis.

Detection of Cleaved Caspase-3 by Confocal Microscopy

The cells were grown in two chambered cell culture slides (BD Biosciences, Bedford, MA), treated with WIN-55,212-2 (5.0, 7.5, 10.0 µM doses) for 24 h, washed with 1x PBS at room temperature and were immediately fixed in cold 100% methanol at -20 ^{o}C for 10 minutes. Cells were blocked with blocking buffer (5.5% normal goat serum in TBST, 50mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100) for 60 min and were washed with TBS (50mM Tris-HCl (pH 7.4), 150 mM NaCl). Cells were then incubated with primary antibody Alexa Fluor 488 conjugate, (Cell Signaling Technology, Beverly, MA) overnight using vendor's protocol. After incubation, cells were washed twice for 5 minutes with TBST and once with TBS. Coverslips were mounted using the Prolong Antifade kit obtained from Molecular Probes, (Eugene, OR). Cells were visualized with a Bio-Rad MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200 microscope at the Keck Neural Imaging Lab in the University of Wisconsin, Madison.

Silencing of ERK 44/42 by Small Interfering RNA

For suppressing ERK1/2 expressions, ERK1, ERK2 and control scrambled siRNA were purchased from Cell Signaling Technology (Danvers, MA). LNCaP cells were transfected with siRNAs (ERK1, 150 nmol/L and ERK2, 80 nmol/L, scrambled SiRNA 150 nmol/L) using nucleofection kit R specific for LNCaP transfection from Amaxa Biosystems (Gaithersburg, MD). Cells were resuspended in a solution from nucleofector kit R following the manufacturer guidelines. 100 µl of nucleofector solution R was mixed with 2 x 10^6 cells and siRNA. It was then transferred to the cuvette provided with the kit and was nucleofected with an Amaxa Nucleofector apparatus. Cells were transfected using the T-001 pulsing parameter and were transferred into 100 mm plates containing 37 ^{o}C pre warmed culture medium. After transfection, cells were cultured for 48 h, after which the media was replaced with fresh media and cells were treated with 7.5 µM WIN-

55,212-2 for 24 h and protein lysates were prepared. Using 2 μg of GFP we observed 70-80% transfection efficiency with this protocol.

Preparation of Cell Lysates and Western Blot Analysis

Following treatment of cells with WIN-55,212-2, the medium was aspirated and the cells were washed with cold PBS (10 mmol/l, pH 7.45). The cells were then incubated in ice cold lysis buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l EDTA, 20 mmol/l NaF, 100 mmol/l Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1mmol/l phenyl methyl sulfonyl fluoride (PMSF) (pH 7.4), with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 20 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21.5 G needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14000 x g for 15 minutes at 4 ^{o}C, and the supernatant (total cell lysate) collected, aliquoted and was used on the day of preparation or immediately stored at -80 ^{o}C for use at a later time. For western blotting, 25-50 μg protein was resolved over 12 % polyacrylamide gels and transferred onto a nitrocellulose membrane. The non-specific sites on blots were blocked by incubating in blocking buffer (5% non fat dry milk/1% Tween 20 in 20 mmol/l TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate monoclonal primary antibody in blocking buffer for 90 minutes to overnight at 4 ^{o}C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horse-radish peroxidase conjugate and detected by chemiluminescence and autoradiography using hyperfilm obtained from Amersham Biosciences (UK Ltd.). Densitometric measurements of the bands in western blot analysis were performed using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT).

Statistical Analysis

Results were analyzed using a two-tailed Student's *t* test to assess statistical significance. Values of $P < 0.05$ were considered statistically significant.

Animals

Athymic (*nu/nu*) nude mice (male; 6–8 weeks old) were obtained from NxGen Biosciences (San Diego, CA) and housed in the University of Wisconsin, Medical School Animal Care Facility at standard conditions (in laminar airflow cabinets under pathogen-free conditions with 12 h light/12 h dark schedule) and fed with autoclaved Harlan Teklad sterilizable rodent diet (Madison, WI) *ad libitum*.

In Vivo Tumor Xenograft Model

Athymic (*nu/nu*) male nude mice were obtained from NxGen Biosciences (San Diego), housed under pathogen-free conditions with a 12-h light/12-h dark schedule, and fed with an autoclaved diet ad libitum. We chose CWR22Rv1 cells for determining the *in vivo* effects of WIN-55,212-2 based on the fact that these cells form rapid and reproducible tumors in nude mice and secrete significant amounts of PSA in the bloodstream of the

host. CWR22Rv1 cells were harvested by trypsinization and resuspended in RPMI medium 1640. To establish CWR22Rv1 tumor xenografts in mice, 6- 8 week old athymic mice were injected s.c. with 1×10^6 cells mixed with 50 μl of RPMI plus 50 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA).

Protocol-1

Twenty-four animals were then randomly divided into three groups consisting of eight animals each. The first group of animals received no treatment and served as controls. The animals of groups II and III received an i.p. injection of WIN-55,212-2 (0.5 mg b.wt.) in DMSO and PBS. The treatment schedule was continued until the tumors reached a volume of 1200 mm^3 in control group. At this point, the animals from group 1 and group 2 were withdrawn from the study and euthanized. In group 3 WIN-55,212-2 treatment was continued till tumor volume reached 1200 mm^3. These treatments were repeated thrice a week. Tumor sizes were measured twice weekly, and tumor volume was calculated by the formula $0.5238 \times L_1 \times L_2 \times H$ where L_1 is the long diameter, L_2 is the short diameter, and H is the height of the tumor. Every week, blood samples were collected by the "mandibular bleed" and sera were separated from the whole blood and stored at -80°C until assayed for PSA by specific immunoassay.

Protocol-2

This protocol was designed to assess the therapeutic potential of WIN-55,212-2. For this purpose, CWR22Rv1 cells were inoculated onto the flanks of athymic nude mice as described under protocol 1. The animals were given autoclaved diet and water *ad libitum* until the tumors were established to a volume of 400 mm^3. At this point, the animals were evenly divided into two groups and treatment was started. The details of treatment are as follows.

Group I: control-cells were implanted (at the start of the experiment; Day 0), no further treatment was given; *Group II:* cells were implanted and tumors were allowed to grow to a volume of 400 mm^3, followed by i.p. injection of WIN-55,212-2 (0.5 mg b.wt.) three times a week. The treatment was continued until the tumor reached to a volume of 1200 mm^3. At this point, the animals were withdrawn from the study and euthanized.

Collection of blood and determination of serum prostate specific antigen levels

Blood was collected by 'madibular bleed' and serum was separated by allowing the blood to clot at room temperature for about an hour followed by centrifuging it for 10 min at 4°C. The levels of PSA were determined by using a quantitative human PSA enzyme-linked immunosorbent assay (ELISA) kit (Anogen, Ontario, Canada) as per the manufacturer's protocol. For PSA determination, serum was used within 24–48 h of isolation or samples were stored at –80°C till further use.

Immunohistochemical Analysis

Sections (5µm) were cut from paraffin-embedded tumors. Immunostaining was performed using specific antibodies with appropriate dilutions and was replaced with either normal host serum or block for negative controls, followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counter stained with hematoxylin. The stained slides were dehydrated and mounted in permount and visualized on a Zeiss-Axiophot DM HT microscope (Zeiss-Axiophot, Jena, Germany). Images were captured with an attached camera linked to a computer.

Statistical Analysis

All statistical analyses were carried out with prism (GraphPad, San Diego), and *P* values <0.05 were considered significant.

Results

(1) Expression of Cannabinoid Receptor in Normal and Prostate Cancer Cells

We first compared the expression level of both cannabinoid receptors CB_1 and CB_2 in PrEC and a series of human prostate cancer cells. We also included a pair of cells PZ-HPV-7 (virally transformed cells, derived from normal human prostate tissue) and CA-HPV-10 (virally transformed cells, derived from adenocarcinoma of human prostate tissue) derived from the same individual. Immmunoblot data shown in Fig 1 revealed that expression of both CB_1 and CB_2 receptors was significantly higher in prostate cancer cells LNCaP, DUI45, PC3, CWR22Rv1 and CA-HPV-10 as compared to normal prostate cells PZ-HPV-7 and PrEC cells.
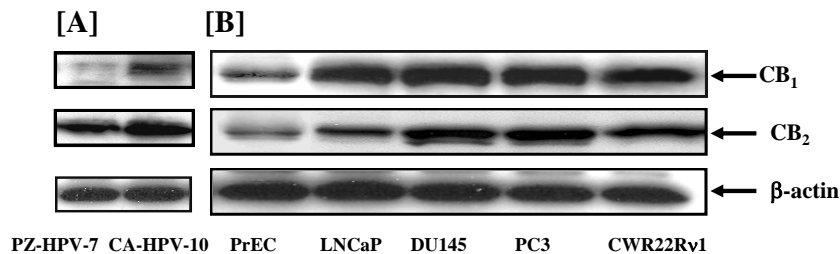

Fig 1 Western Blot analysis of CB_1 and CB_2 cannabinoid receptor expression in normal and human prostate cancer cells. (A), a pair of normal (PZ-HPV-7) and prostate cancer (CA-HPV-10) cells obtained from same individual and (B), PrEC (normal prostate epithelial cells) and prostate cancer cells, LNCaP, DU145, PC-3 and CWR22Rv1. Total cell lysates were prepared and 30 µg protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. β-actin was used as a loading control.

### (2) Effect of WIN-55,212-2 on Cell Viability of PrEC and LNCaP Cells

To evaluate the cell viability response of WIN-55,212-2 on PrEC and LNCaP cells, MTT assay was employed. Data in Fig 2 shows that treatment of PrEC cells with WIN-55,212-2 (1-10 µM) for 24 and 48 h was without effect on cell viability (Fig 2). However, treatment of LNCaP cells with similar doses of WIN-55,212-2, in a dose-dependent manner significantly decreased the viability of LNCaP cells in 24 and 48 hr (Fig 2). The IC_{50} for inhibition of cell viability at 24 and 48 h was 6.0 µM and 5.0 µM respectively.
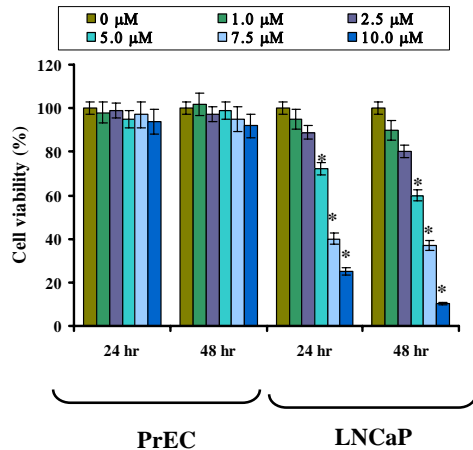
Fig 2 *Effect of WIN-55,212-2 on cell viability of PrEC and LNCaP cells. As detailed in "Materials and Methods," the cells were treated with WIN-55,212-2 (1-10 μM) for 24 h and 48 h, and their viability was determined by MTT assay. The data are expressed as percent cell viability and represent means ± SE of three separate experiments in which each treatment was performed in 10 wells.* *p<0.001 compared to control (0 μM).*

(3) CB$_1$ and CB$_2$ receptor activation signals growth inhibition in LNCaP cells

To study the possible implication of CB$_1$ and CB$_2$ receptor in WIN-55,212-2 induced cell death, the effect of their antagonists was evaluated using MTT assay. Cells pretreated with 2 μM of SR141716 (CB$_1$ antagonist) or SR144528 (CB$_2$ antagonist) had no effect on cell viability but exhibited significant protective effect when co-administered with WIN-55,212-2 (7.5 μM) at a molar ratio of 1:3.75 (Fig 3). These data suggest that both CB$_1$ and CB$_2$ receptors may be involved in WIN-55,212-2 mediated growth inhibition and apoptosis.
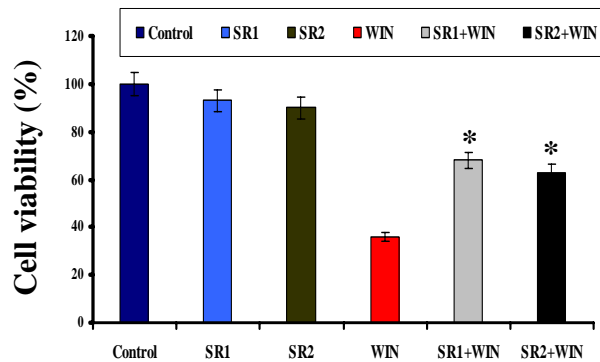

Fig 3 *Effect of CB$_1$ receptor antagonist SR141716 (SR1) and CB$_2$ receptor antagonist SR144528 (SR2) on WIN-55,212-2 induced cell viability. As detailed in "Materials and Methods," cells were treated with 2 μM of SR141716 or SR144528 alone for 24 h. In another parallel set, cells were pretreated with each of these antagonists for 3 h followed by incubation with 7.5 μM WIN-55,212-2 for 24 h and their viability was determined by MTT assay. The data are expressed as percent cell viability and represent means ± SE of three separate experiments in which each treatment was performed in 10 wells.* *p< 0.001 compared to WIN.*

(4) Effect of WIN-55,212-2 on Apoptosis and Necrosis of LNCaP Cells

We next assessed whether the cell growth inhibitory effect of WIN-55,212-2 was associated with induction of apoptosis. The induction of apoptosis by WIN-55,212-2 was evident from the analysis of the data obtained by confocal microscopy after labeling the cells with annexin V (Fig 4A). This method was used as it identifies the apoptotic (*green fluorescence*) as well as necrotic (*red fluorescence*) cells. As shown by the data WIN-55,212-2 treatment resulted in a dose-dependent apoptosis in LNCaP cells. We next quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with

deoxyuridine triphosphate and PI. LNCaP cells were treated with of WIN-55,212-2 (1-10 μM) for 24 h. As shown by data in Fig 4B, WIN-55,212-2 treatment resulted in 18.3 and 25.6% of apoptotic cells at a dose of 7.5 and 10 μM, respectively. While the induction of apoptosis was almost negligible at the lowest dose (1.0 μM) used, the highest dose (10 μM) employed resulted in a massive induction of apoptosis as determined by flow cytometry.

(A)   **(B)**
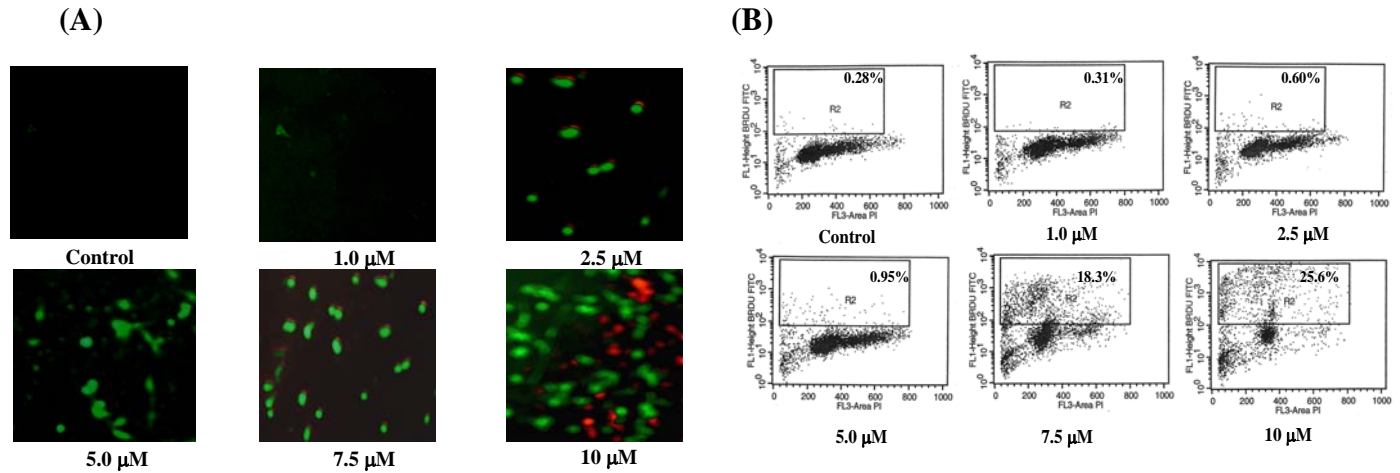
Fig 4 *(A) Induction of apoptosis by confocal microscopy; cells were treated with WIN-55,212-2 1-10 μM for 24 h for dose-dependent study. The Annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells. This kit uses a dual-staining protocol in which apoptotic cells are stained with annexin V (green fluorescence) and necrotic cells are stained with PI (red fluorescence). (B), quantification of apoptosis by flow cytometry. The cells were treated with WIN-55,212-2 (1-10 μM) for 24 h, labeled with deoxyuridine triphosphate using terminal deoxynucleotide transferase and PI. Cells showing fluorescence (R2) are considered as apoptotic cells and their percentage population is shown in the figure. Data shown are from a representative experiment repeated three times with similar results.*
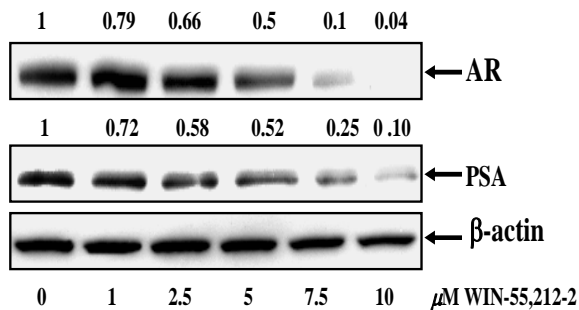
(5) Effect of WIN-55,212-2 on AR and PSA Protein and mRNA Expression in LNCaP Cells

Androgens are involved in the development and progression of prostate cancer where AR is assumed to be the essential mediator for androgen action (17, 18). In the next series of experiments, we determined the effect of WIN-55,212-2 on protein and mRNA expression of AR. In dose-dependent study, we found that treatment of LNCaP cells with WIN-55,212-2 resulted in a marked decrease in AR protein expression (Fig 5A). Relative density data of these immunoblots revealed that the decrease in AR protein expression was 50% and 90 % at 5.0 and 7.5 μM of WIN-55,212-2, respectively. In time-dependent study with 5 μM dose of WIN-55,212-2, there was a marked decrease in AR protein expression and this corresponded with the relative density data showing a decrease of 61% and 69% at 48 h and 72 h, respectively (Fig 5B). Studies have also shown that modulation in AR leads to alteration in androgen-responsive genes (19). PSA is an androgen responsive gene and is regarded as the most sensitive biomarker and screening

tool for prostate cancer in humans (20). The dose-dependent effect of WIN-55,212-2 on LNCaP cells showed a significant decrease in PSA protein expression at 5.0, 7.5 and 10 μM concentrations when assessing at 24 h post treatment (Fig 5A). Densitometric analysis data revealed that the decrease was 48%, 75% and 90% at 5.0, 7.5 and 10.0 μM concentrations (Fig 5A). For time-dependent study cells were treated with 5 μM of WIN-55,212-2 for 24, 48 and 72 h. Employing western blot analysis we found a significant decrease in a time-dependent manner in PSA protein expression. Densitometric analysis revealed a decrease in PSA protein expression by 48% and 60% at 48 and 72 h respectively (Fig 5B). We also evaluated the effect of WIN-55,212-2 on mRNA levels of AR and PSA. As shown by the real time-PCR analysis data, there was an inhibition in mRNA levels of AR (Fig 5C) and PSA (Fig 5D) at 7.5 μM and 10.0 μM concentrations.

We next examined the effect of WIN-55,212-2 on secreted levels of PSA in LNCaP cells. Employing ELISA technique, we found that treatment of LNCaP cells with WIN-55,212-2 resulted in a dose-dependent decrease in the secreted levels of PSA by 30%, 53% and 62% at 5.0, 7.5 and 10 μM, respectively. At similar doses of WIN-55,212-2 but varying the time-point by 48h, PSA levels decreased by 53%, 77% 80% (Fig 5E). Further at 72 h post treatment of WIN-55,212-2 secreted PSA levels decreased by 58%, 82% & 88% (Fig 5E). From these data it seems that the decrease in LNCaP cell growth was concomitant with a decrease in AR protein expression as well as decrease in both intracellular and secreted PSA levels.
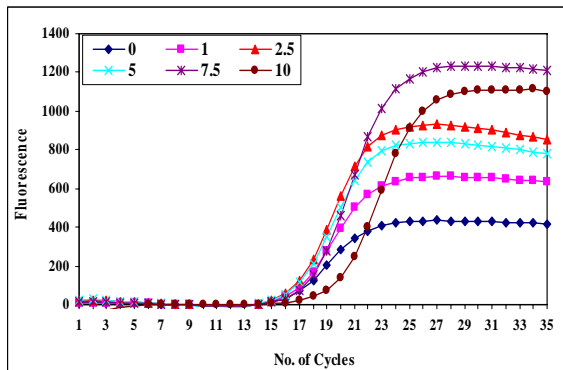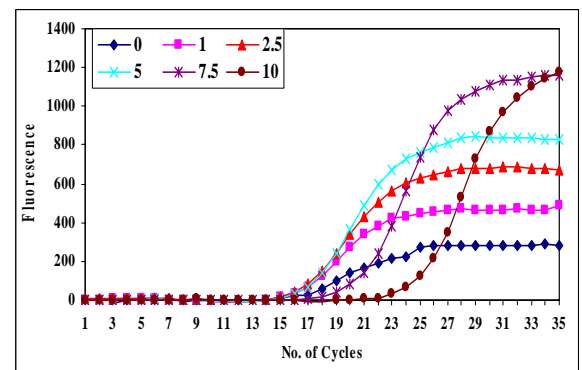
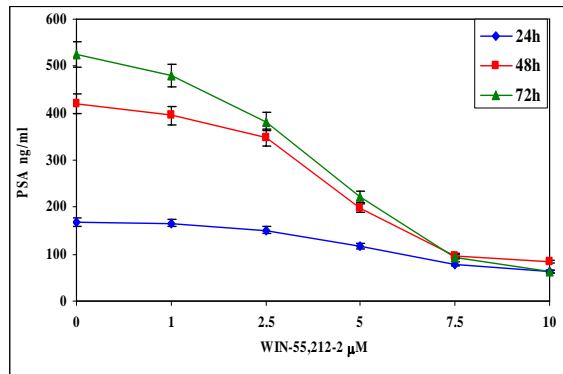**[A] Dose-dependent**
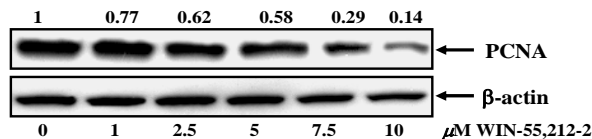
[B] Time-dependent
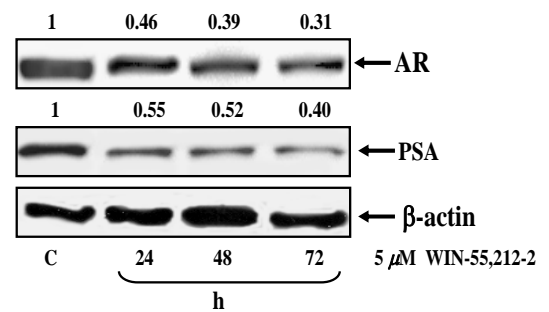
(C)

(D)

(E)

Fig 5 Effect of WIN-55,212-2 on protein and mRNA expression of AR and PSA in LNCaP cells. (A), dose-dependent effect and (B), time-dependent effect. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μg protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the figures represent change as compared to vehicle treatment, in protein expression of the bands normalized to β-actin. Western blot data shown here are from a representative experiment repeated three times with similar results. (C) and (D), effect of WIN-55,212-2 on mRNA expression of AR (C), and PSA (D) determined by real time-PCR. PCR data shown here are from a representative experiment repeated twice with similar results. (E), effect on secreted levels of PSA. Cells were treated with WIN-55,212-2 (1-10 μM) for 24, 48 and 72 h and then harvested. The PSA levels were determined by ELISA as described under "Methods". The data in E represents the mean ± SE. of three independent experiments.

(6) Effect of WIN-55,212-2 on Cell Proliferation Marker, PCNA

We next determined the effect of WIN-55,212-2 on PCNA that serves as a requisite auxiliary protein for DNA polymerase δ-driven DNA synthesis and is cell regulated (21, 22). In the dose-dependent study treatment of LNCaP cells with WIN-55,212-2 (1-10 μM) resulted in a significant decrease in protein expression of PCNA. Western blot analysis and relative density of these bands showed that the decrease in protein expression of PCNA was 71% at 7.5 μM WIN-55,212-2 (Fig 6A). In time-dependent study, treatment of LNCaP cells with 5 μM WIN-55,212-2 resulted in greater than 50% inhibition in PCNA protein expression at 48 and 72 h of treatment (Fig 6B).
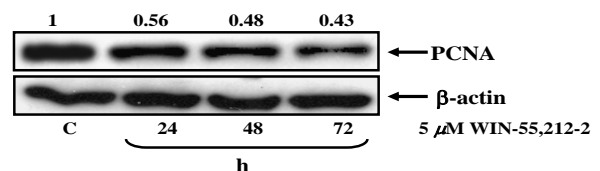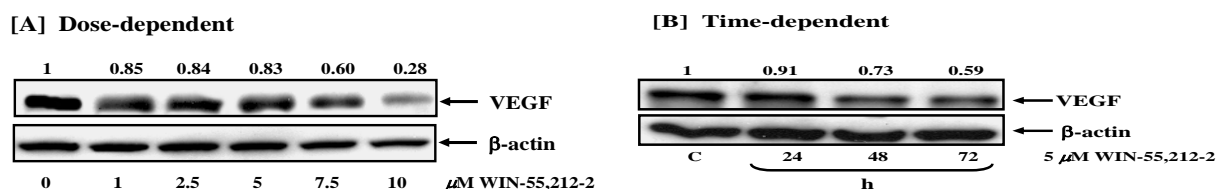

Fig 6 Effect of WIN-55,212-2 on protein expression of PCNA in LNCaP cells. (A), dose-dependent effect, (B), time-dependent effect. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μg protein was subjected to SDS-PAGE,

followed by immunoblot analysis and chemiluminescence detection. The values above the figures represent change as compared to vehicle treatment, in protein expression of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

(7) Effect of WIN-55,212-2 on Vascular Endothelial Growth Factor (VEGF)

Because VEGF is a marker for angiogenesis it is considered that blocking the angiogenic process may represent a promising way of treating tumor. Studies have shown that androgen regulates VEGF content in prostate cancer (23). As WIN-55,212-2 treatment resulted in a decrease in AR expression the effects on VEGF were also determined. It was observed that WIN-55,212-2 treatment also resulted in a decrease in VEGF protein expression (Fig 7A). Densitometric analysis data showed a decrease of 40% at 7.5 μM concentration of WIN-5,212-2. In time-dependent study at 5 μM WIN-55,212-2 treatment VEGF protein expression decreased in a time-dependent manner (Fig 7B).

[A] Dose-dependent

| 1 | 0.85 | 0.84 | 0.83 | 0.60 | 0.28 |

VEGF

β-actin

| 0 | 1 | 2.5 | 5 | 7.5 | 10 | μM WIN-55,212-2 |

[B] Time-dependent

| 1 | 0.91 | 0.73 | 0.59 |

VEGF

β-actin

| C | 24 | 48 | 72 | 5 μM WIN-55,212-2 |
h

Fig 7 Effect of WIN-55,212-2 on protein expression of VEGF in LNCaP cells. (A), dose-dependent effect, (B), time-dependent effect. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μg protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the figures represent change as compared to vehicle treatment, in protein expression of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

(8) WIN-55,212-2 causes G$_1$ phase cell cycle arrest

We have earlier shown that treatment of LNCaP cells with WIN-55,212-2 (1−10 μM) for 24 h significantly decreased the cell viability and led to induction of apoptosis (15). Several studies have shown that the induction of apoptosis may be cell cycle dependent (24-28). Therefore, in next series of experiments, we tested the hypothesis that WIN-55,212-2 caused apoptosis of LNCaP cells is mediated via cell cycle blockade. We performed DNA cell cycle analysis to assess the effect of WIN-55,212-2 treatment on the distribution of cells in the cell cycle. As shown in Fig. 8, compared with vehicle treatment, WIN-55,212-2 treatment resulted in a dose-dependent accumulation of cells in G$_1$ phase of the cell cycle (59%, 62%, 69%, 81% and 83% cells in G$_1$ phase at 1.0, 2.5, 5.0, 7.5 and 10 μM concentrations, respectively). This observation is important because the molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (29, 30). Consistent with this

observation, in recent years, inhibition of the cell cycle has been appreciated as a target for the management of cancer (31, 32).
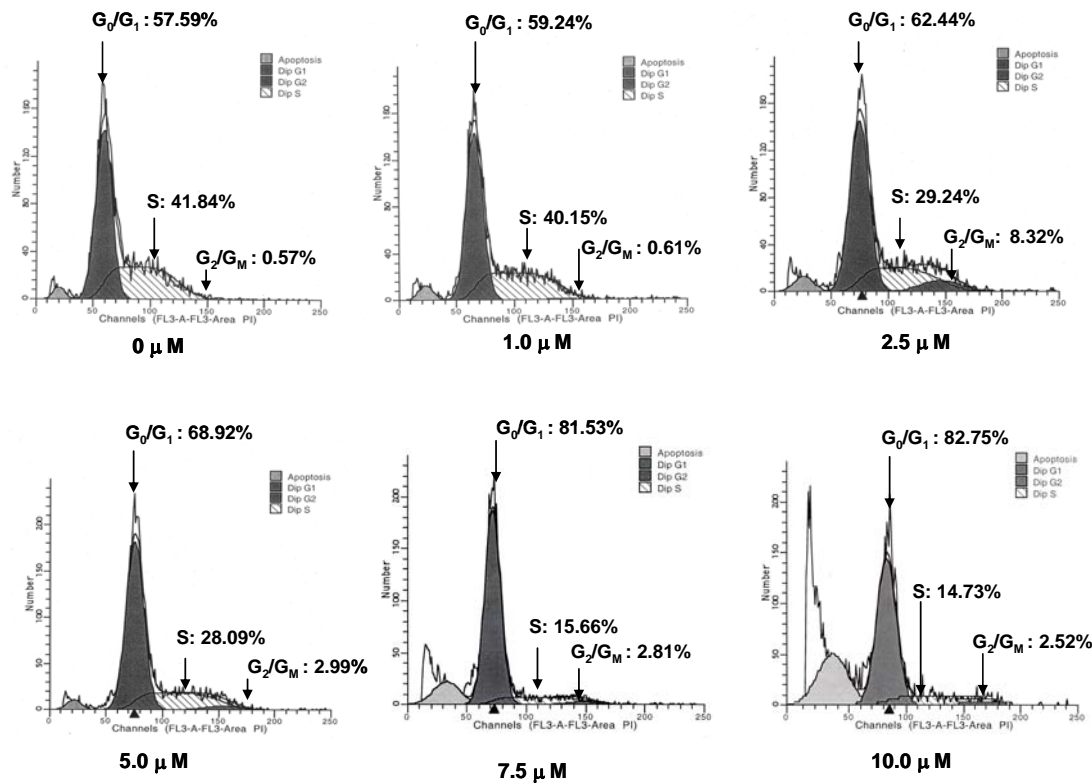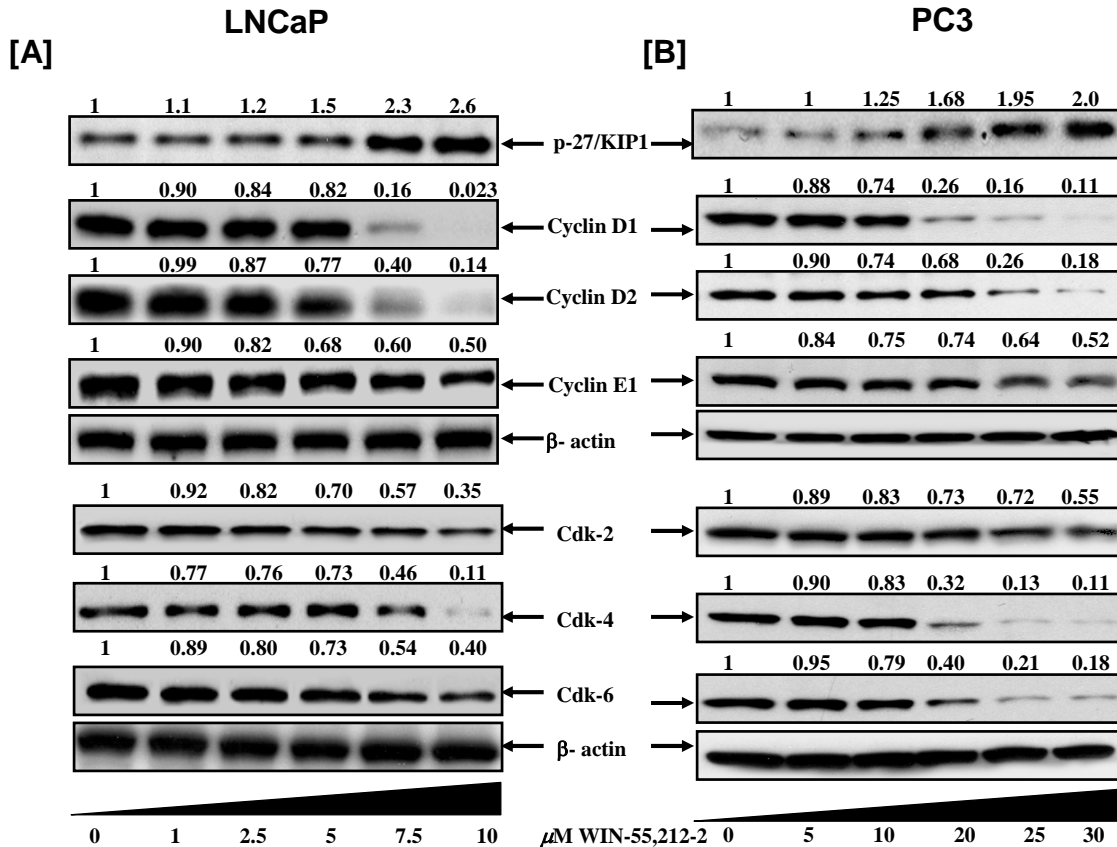

Fig 8: Effect of WIN-55,212-2 treatment on cell cycle in LNCaP cells. Cell cycle analysis was performed by flow cytometry as detailed in Materials and Methods. The labeled cells were analyzed using a FACScan benchtop cytometer and percentage of cells in G_0–G_1, S and G_2-M phase were calculated using ModFit LT software. The data shown here are from a typical experiment repeated three times.

(9) WIN-55,212-2 induced cell cycle arrest is mediated via an induction of KIP1/p27 and concomitant inhibition in cyclins D1, D2, E and cdk2, cdk4 and cdk6

Because our studies demonstrated that WIN-55,212-2 treatment of cells resulted in a G_1-phase cell cycle arrest and apoptosis, we examined the effect of WIN-55,212-2 on cell cycle-regulatory molecules operative in G_1 phase of the cell cycle. Studies have shown a critical role of p27/KIP1 in apoptosis and cell cycle progression through G_0-G_1 phase (33-35). We observed a significant induction of p27/KIP1 by WIN-55,212-2 at 5-10 μM doses. (Fig.9A). Relative density data revealed 1.5, 2.3 and 2.6 fold increase in the protein expression of Kip/p27 at 5.0, 7.5 and 10 μM concentrations of WIN-55,212-2 respectively. Using immunoblot analysis, we also assessed the effect of WIN-55,212-2 treatment on the protein expression of the cyclins and cdks, which are known to be regulated by KIP1/p27. WIN-55,212-2 treatment of the cells resulted in a dose-dependent decrease in protein expression of cyclin D1, cyclin D2, and cyclin E (Fig. 9A) as well as cdk2, cdk4, and cdk6 (Fig. 9A) . Densitometric analysis data of cyclins

revealed a significant decrease in the expression of cyclin D1 (84%, 97%), cyclin D2 (60%, 86%) and cyclin E (40%, 50%) at 7.5 and 10.0 μM concentrations of WIN-55,212-2 respectively (Fig. 9B). Relative density data of cdks also revealed a significant decrease in the expression of cdk2 (43%, 65%), cdk4 (54%, 89%) and cdk6 (46%, 60%) at similar doses of WIN-55,212-2. In the next series of experiments we assessed the effect of WIN-55,212-2 on p27/KIP1, cyclin's and cdk's in androgen insensitive cell PC3. Cells were treated with different doses of WIN-55,212-2 (5, 10, 20, 25, 30 μM) and we found an induction in p27/KIP1 and down regulation in the protein expression of cyclin's and cdk's particularly at doses of 20-30 μM (Fig 9B).

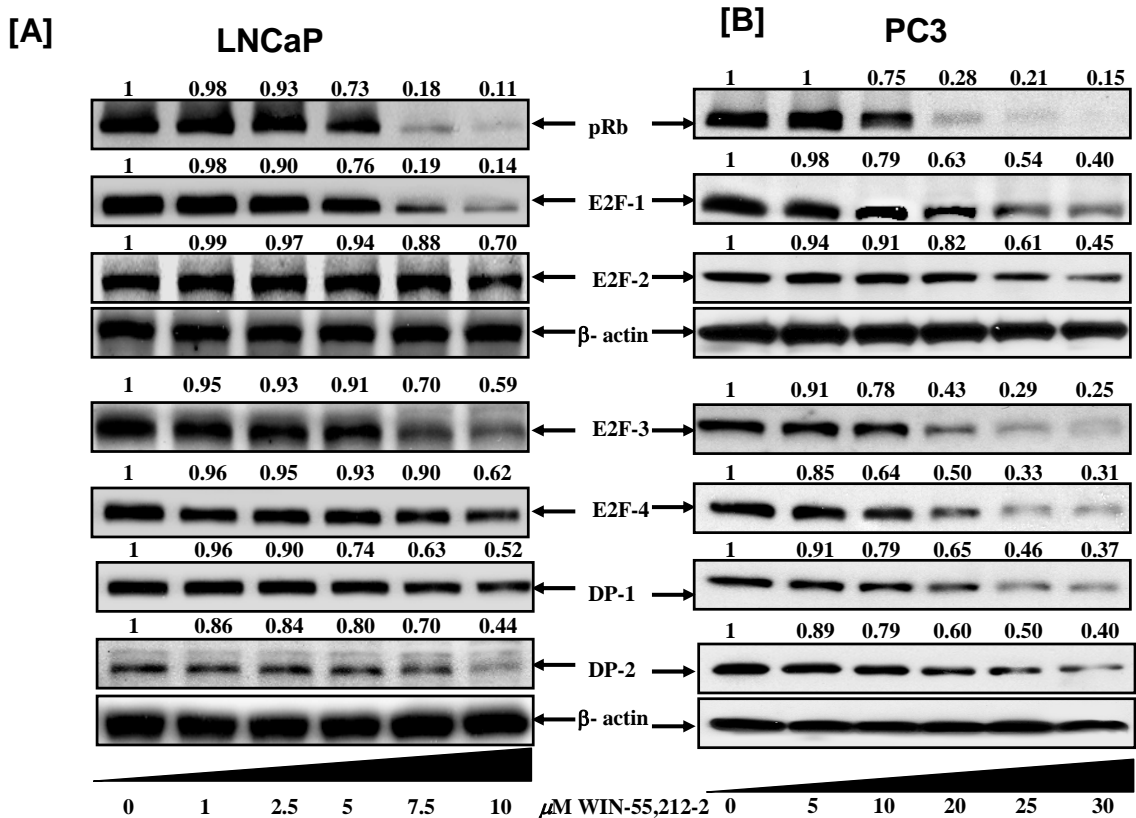*Fig 9: (A) Effect of WIN-55,212-2 treatment on the protein expression of KIP1/p27, cyclin D1, D2 and E and cdk 2, 4 & 6 in LNCaP cells. (B) Effect of WIN-55,212-2 treatment on the protein expression of KIP1/p27, cyclinD1, D2 and E and cdk 2, 4 & 6 in PC3 cells. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 and total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.*

(10) WIN-55,212-2 inhibits protein expression of pRB, E2F and DP

Downregulation of cdk4/6 has been shown to be associated with a decrease in the expression of retinoblastoma (pRb) tumor suppressor protein a key regulator of the $G_1 \rightarrow S$ phase transition in the cell cycle (36, 37). Therefore, we next examined the effect

of WIN-55,212-2 on protein expression of pRb. Immunoblot data revealed that WIN-55,212-2 treatment of cells resulted in a significant decrease in the protein expression of pRb. Densitometric analysis of immunoblots showed 27%, 82% and 89% inhibition at 5.0, 7.5 and 10 μM concentrations of WIN-55,212-2 (Fig. 10A). Since pRb controls cell cycle by binding to and inhibiting the E2F transcription factors, we determined the protein expression of E2F (1-4) transcription factors. As shown in Fig. 10A, WIN-55,212-2 treatment of cells resulted in a dose-dependent decrease in E2F transcription factors. Relative density data revealed an inhibition in E2F-1 (81% and 86%), E2F-2 (12% and 30%), E2F-3 (30% and 41%) and E2F-4 (10% and 38%) at a concentration of 7.5 μM and 10 μM of WIN-55,212-2. Since the activity of E2F is known to be dependent on its heterodimeric association with members of DP family of proteins, we also evaluated the effect of WIN-55,212-2 treatment on both members of DP family viz. DP-1 and DP-2. Immunoblot and densitometric analysis data revealed a decrease in the protein expression of DP-1 (37% and 48%) and DP-2 (30% and 56%) at 7.5 μM and 10 μM concentration of WIN-55,212-2 (Fig. 10A). In the next series of experiments we assessed the effect of WIN-55,212-2 on pRB, E2F family of proteins (1-4) and its heterodimeric partners DP-1 and DP-2 in androgen insensitive cell PC3. Cells were treated with different doses of WIN-55,212-2 (5, 10, 20, 25, 30 μM) and we found a decrease in the protein expression of pRB, E2F (1-4), DP-1 and DP-2 at 20-30 μM of doses (Fig 10B).
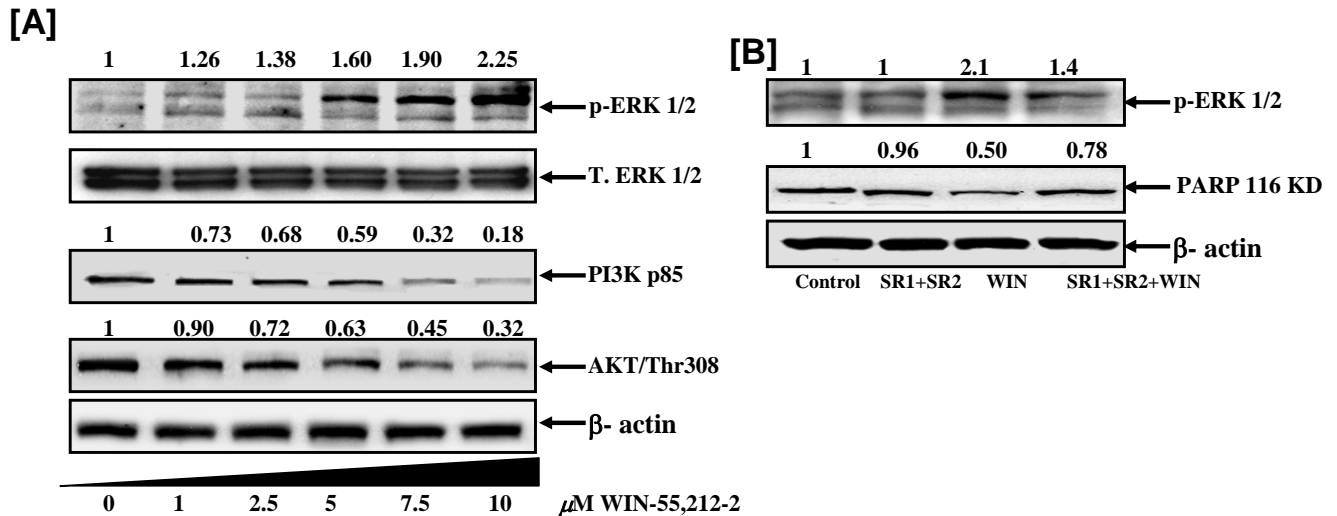

Fig 10: *Effect of WIN-55,212-2 treatment on protein expression of (A) pRb and E2F (1-4) (B) DP1 and DP2, in LNCaP cells. As detailed in "Materials and Methods" the cells were treated*

with DMSO alone or specified concentrations of WIN-55,212-2 and total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

(11) WIN-55,212-2 induced sustained activation of ERK and inhibition of PI3K/AKT leads to apoptosis through cannabinoid receptors

It has been reported that challenging gliomas with cannabinoids leads to the activation of ERK1/2 signaling and AKT inhibition (38, 3). This sustained ERK1/2 activation can mediate cell-cycle arrest (1). We observed a significant and sustained activation of ERK1/2 and significant inhibition of PI3K (p85) and AKT (Thr[308]) when LNCaP cell were treated with WIN-55,212-2 at a dose of 1-10 μM (Figure 11A). To confirm that ERK1/2 activation is cannabinoid receptor mediated, cells were pretreated with 3 μM of SR141716 (CB_1 antagonist) and SR144528 (CB_2 antagonist) for 3 h followed by treatment with WIN-55,212-2. Data in Fig. 11B show that there was no activation of ERK1/2 when treated with the antagonists alone. WIN-55,212-2 (7.5 μM) treatment resulted in significant activation of ERK1/2. When antagonist were coadministered with WIN-55,212-2 there was a decrease in the protein expression of ERK1/2 and a significant increase in the protein expression of PARP (116 KD) as compared to the treatment 7.5 μM WIN-55,212-2 alone (Fig. 11B). These data suggest that sustained ERK1/2 activation and subsequent apoptosis is mediated through cannabinoid receptors.

Fig 11: *Effect of WIN-55,212-2 treatment on protein expression of (A) ERK1/2 (phospho-p44/42, Thr[202]/Tyr[204]), Anti-PI3K kinase (p85) and AKT (Thr[308]) in LNCaP cells (B) Effect of CB_1 (SR141716) and CB_2 (SR144528) antagonist on protein expression of ERK1/2 and PARP cleavage (116KD) in LNCaP cells. As detailed in "Materials and Methods" the cells were treated with 7.5 μM concentrations of WIN-55,212-2 and 3 μM of CB_1 (SR141716) and CB_2 (SR144528). Total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin using UN-SCAN-IT software (Silk Scientific,*
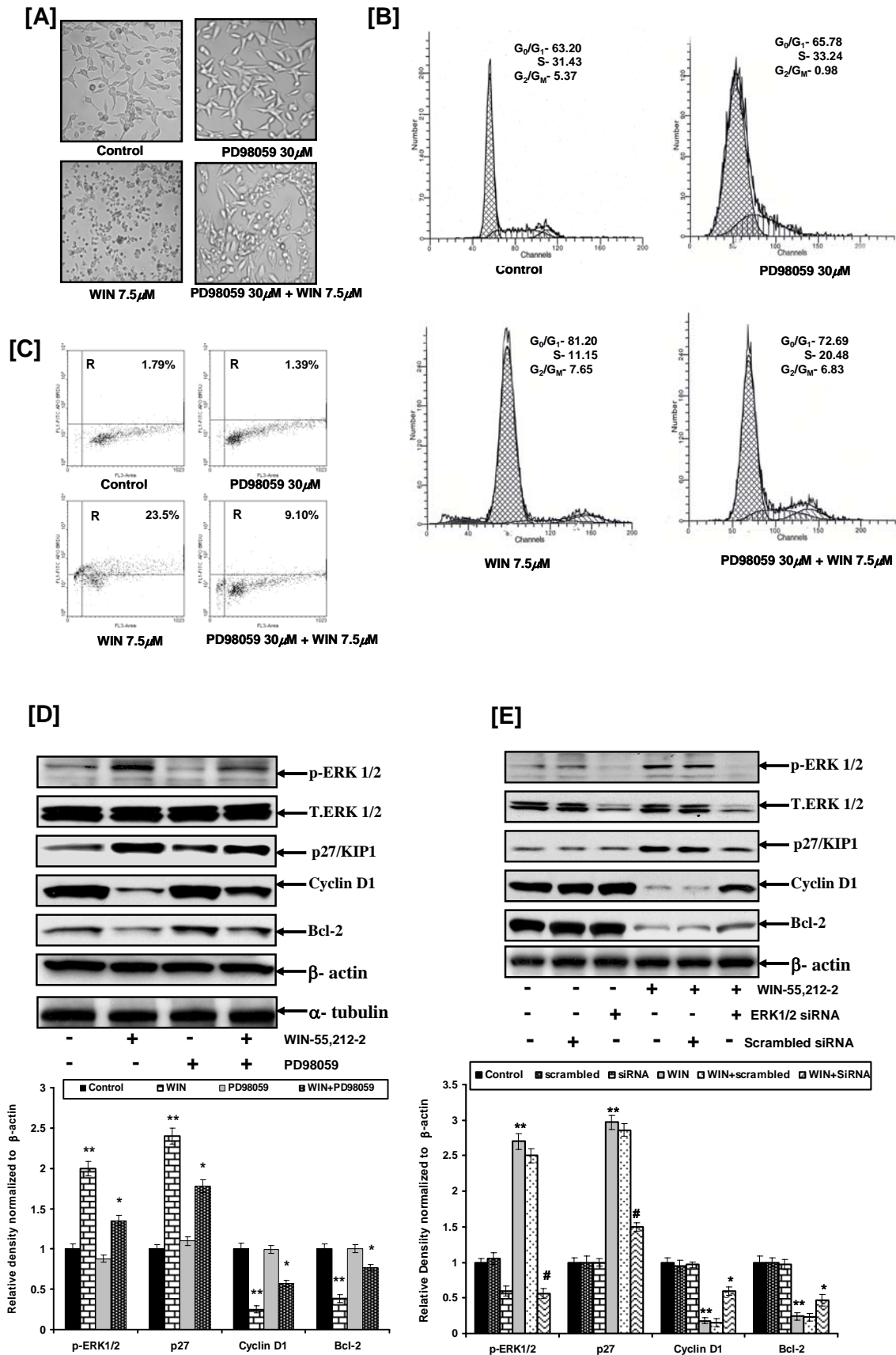
Inc., Orem, UT). The data shown here are from a representative experiment repeated three times with similar results.

(12) WIN-55,212-2 induced sustained activation of ERK1/2 leads to cell growth inhibition with the induction of apoptosis and cell cycle arrest

To define the role of ERK1/2 in cannabinoid receptor-induced cell growth inhibition and apoptosis, LNCaP cells were pretreated with ERK1/2 inhibitor PD98059 (30 µM) for 1 h. This treatment alone resulted in no change in the morphology of the cells. However, 7.5 µM WIN-55,212-2 treatment resulted in distinct morphological changes in LNCaP cells, as cells became round and detached from the surface of the plate, whereas pretreatment of LNCaP cells with PD98059 (30 µM) prevented these morphological changes (Fig. 12A). WIN-55, 212,2 treatment of LNCaP cells results in G_1 cell cycle arrest. To assess whether cell cycle arrest is mediated via activation of ERK1/2, we next performed DNA cell cycle analysis. As shown in Fig 12B, blocking of ERK1/2 activation by its inhibitor PD98059 resulted in decrease in the number of cells in the G_1 phase of cell cycle (72%) when compared to WIN-55,212-2 treatment alone (81%). To assess whether cell cycle dysregulation leads to induction of apoptosis we next quantified the extent of apoptosis by flow cytometric analysis. As shown in Fig 12C WIN-55,212-2 treatment of LNCaP cells at a dose of 7.5 µM resulted in 23% of apoptotic cells. Apoptosis was only 9% when WIN-55,212-2 (7.5 µM) was coadministered with PD98059 (30 µM). We next determined whether PD98059 reversed the activation of ERK1/2 by WIN-55,212-2 treatment alone and we found that ERK1/2 protein expression was significantly decreased when WIN-55,212-2 was given in combination with PD98059 (Fig 12D). We next determined the effect of PD98059 on p27/KIP1 (Fig 12D), a cell cycle-regulatory molecule operative in G_1 phase of the cell cycle, and cyclin D1 because of its function in influencing cell proliferation. WIN-55,212-2 treatment increased the protein expression of p27/KIP1 whereas this increase in expression was downregulated when WIN-55,212-2 was given in combination with PD98059. WIN-55,212-2 treatment significantly inhibited the expression of cyclin D1 and this effect was significantly reversed (>55 %) when WIN-55,212-2 was coadminstered with PD98059 (Fig. 12D). We also observed a decrease (62%) in the protein expression of BCl-2, a pro-apoptotic protein when the cells were treated with WIN-55,212-2 at 7.5 µM; this effect was significantly reversed to 50% when WIN-55,212-2 was coadministered with ERK1/2 inhibitor (Fig 12D).

To further validate the role of ERK1/2 in WIN-55,212-2 induced cell cycle arrest leading to apoptosis, we silenced ERK1/2 by using small interfering RNA against ERK1/2. We observed that WIN-55,212-2 did not induce ERK1/2 activation and p27 when ERK1/2 was silenced (Fig 12E). Similarly protein expression of cyclin D1 and Bcl-2 which downregulated by WIN-55,212-2 was found to be reversed when ERK1/2 was silenced.

[A] Control, PD98059 30μM, WIN 7.5μM, PD98059 30μM + WIN 7.5μM

[B]

Control — G₀/G₁- 63.20, S- 31.43, G₂/GM- 5.37

PD98059 30μM — G₀/G₁- 65.78, S- 33.24, G₂/GM- 0.98

WIN 7.5μM — G₀/G₁- 81.20, S- 11.15, G₂/GM- 7.65

PD98059 30μM + WIN 7.5μM — G₀/G₁- 72.69, S- 20.48, G₂/GM- 6.83

[C] Control R 1.79%, PD98059 30μM R 1.39%, WIN 7.5μM R 23.5%, PD98059 30μM + WIN 7.5μM R 9.10%

[D] p-ERK 1/2, T.ERK 1/2, p27/KIP1, Cyclin D1, Bcl-2, β-actin, α-tubulin; WIN-55,212-2, PD98059; Control, WIN, PD98059, WIN+PD98059; Relative density normalized to β-actin; p-ERK1/2, p27, Cyclin D1, Bcl-2

[E] p-ERK 1/2, T.ERK 1/2, p27/KIP1, Cyclin D1, Bcl-2, β-actin; WIN-55,212-2, ERK1/2 siRNA, Scrambled siRNA; Control, scrambled, siRNA, WIN, WIN+scrambled, WIN+SiRNA; Relative Density normalized to β-actin; p-ERK1/2, p27, Cyclin D1, Bcl-2

Fig 12: *Effect of simultaneous treatment of WIN-55,212-2 and ERK1/2 inhibitor on (A) the morphology of LNCaP cells (B) cell cycle in LNCaP cells. Cell cycle analysis was performed by flow cytometry as detailed in 'Materials and Methods'. The labeled cells were analyzed using a FACScan benchtop cytometer and percentage of cells in G_0–G_1, S and G_2-M phase were calculated using ModFit LT software. The data shown here are from a typical experiment repeated three times. (C) quantification of apoptosis by flow cytometry. Cells showing fluorescence (R) are considered as apoptotic and their percentage population is indicated. Data from representative experiments repeated thrice with similar results. (D) protein expression of p27/KIP1, cyclin D1 and Bcl-2 in LNCaP cells. As detailed in "Materials and Methods" the cells were treated with 7.5 μM concentrations of WIN-55,212-2 and 30 μM of ERK1/2 inhibitor PD98059. Total cell lysates were prepared for immunoblot analysis. The bar diagram represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results (*P< 0.01 compared with WIN, **P<0.001 compared with control). (E) silencing of ERK1/2 prevents activation of ERK1/2 and p27 and reverses down regulation of cyclin D1 and Bcl-2. LNCaP cells transfected with 150nM of ERK1, 80 nM of ERK2 or scrambled siRNA (150nM) for 48 h and were then treated with 7.5 μM of WIN-55,212-2 for 24. Cell lysates were analyzed by immunoblotting using antibodies against ERK1/2 (phospho-p44/42, Thr^{202}/Tyr^{204}), p27, cyclin D1, Bcl-2 and β-actin. The bar diagram represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results (*P< 0.01, $^{\#}$P<0.001 compared with WIN, **P<0.001 compared with control).*

(13) WIN-55,212-2 induces apoptosis via classical apoptotic pathway

The above data suggest that WIN-55,212-2 induces growth inhibition via cell cycle arrest in G_1 phase of the cell cycle followed by apoptosis. Since p53 is one of the major regulators of apoptosis, expression of this tumor suppressor sensitizes cells to apoptosis in response to stress. We observed a significant upregulation in the protein expression of p53 when cells were treated with WIN-55,212-2 (Fig 13A). p53-induced apoptosis results from overlapping downstream pathways that suppress mitogenic and survival signaling and promote pro-apoptotic signaling. In this context, p53 can up-regulate the pro-apoptotic Bcl-2 family member Bax and possibly transcriptionally repress the anti-apoptotic protein Bcl-2. Since Bax and Bcl-2 plays a crucial role in apoptosis, we next determined the effect of WIN-55,212-2 treatment of LNCaP cells on protein levels of Bax and Bcl-2. The western blot analysis exhibited a significant increase in the protein expression of Bax at 7.5 and 10 μM concentrations of WIN-55,212-2 (Fig. 13A). In sharp contrast, the protein expression of Bcl-2 was significantly decreased by WIN-55,212-2 treatment in a dose-dependent fashion (Fig. 13A). A significant dose-dependent shift in the ratio of Bax to Bcl-2 was observed after WIN-55,212-2 treatment indicating the induction of apoptotic process (Fig. 13B). Relative density data revealed an increase in protein expression of Bax by 2.1 and 2.9 folds with concomitant decrease in Bcl-2 protein expression by 71% and 79% at a dose of 7.5 and 10 μM respectively. Decrease in Bcl-2 expression was associated with an increase in AIF to 2.0 and 2.1 folds at the above mentioned doses of WIN-55,212-2 (Fig. 13A).

Alteration in Bax/Bcl-2 is known to initiate caspase signaling, therefore, we evaluated the involvement of various caspases during WIN-55,212-2-mediated apoptotic death of LNCaP cells. As shown by the immunoblot analysis, WIN-55,212-2 treatment was found

to result in a significant decrease in the pro form of caspase-3 (Fig 13C), caspase-6, caspase-7 and caspase-9 (Fig 13D) at a concentration of 7.5 and 10 μM. To assess possible involvement of caspase-3 activation in apoptosis, we next measured cleaved caspase-3 by immunoblot analysis and immunostaining (Fig 13C). Cells were stained with Alexa Fluor 488 conjugate antibody and were viewed under confocal microscope. Intensity of the active caspase-3 staining was higher in cells treated with 7.5 and 10 μM concentrations of WIN-55,212-2 as compared to that at lower concentrations of WIN-55,212-2 and control (Fig 13C). The downstream signals during apoptosis are transmitted via caspases, which upon conversion from pro to active forms mediate the cleavage of PARP. We found that WIN-55,212-2 treatment caused cleavage of 116 KD PARP to 85 KD (Figure 13E). Relative density data revealed a decrease in the protein expression of PARP (116KD) (49% and 81%) with a concomitant increase in its cleaved product (85KD) by 3.1 and 4.4 folds at concentrations of 7.5 and 10 μM, respectively.
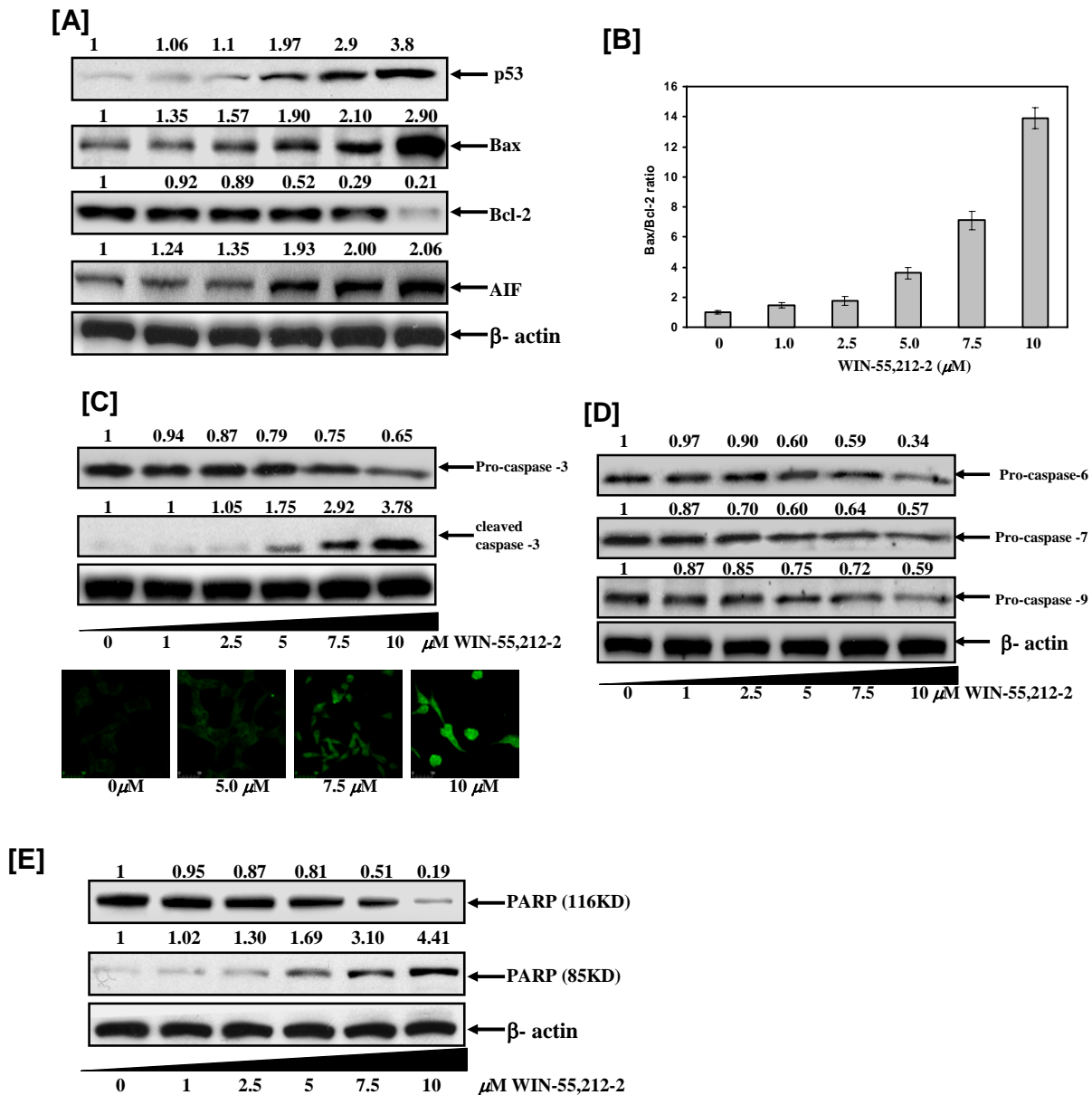
Fig 13: Effect of WIN-55,212-2 treatment on (A) protein expression of p53, Bax, Bcl-2 and AIF (B) Bax/BCl-2 ratio (C) protein expression of pro-caspase-3, cleaved caspase-3 (D) protein expression of pro-caspase 6, 7, 9 (D) and (E) cleavage of PARP. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 and total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results. The data obtained from the immunoblot analyses of Bax and Bcl-2 were used to evaluate the effect of WIN-55,212-2 on the Bax/Bcl-2 ratio. The densitometric analysis of Bax and Bcl-2 bands was performed using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT), and the data (relative density normalized β-actin) were plotted as Bax/Bcl-2 ratio. Detection of cleaved caspase-3 by confocal fluorescence microscopy; cells were treated with WIN-55,212-2 5.0, 7.5 and 10 μM for 24 h and were stained with antibody Alexa Fluor 488 conjugate.

(14) Effect of WIN-55,212-2 on Androgen Receptor and PSA Protein Expression in CWR22Rv1 Cells

We selected androgen-responsive CWR22Rv1 cells for these studies, because they express AR and also secrete PSA in the bloodstream of the host. To accomplish this work, we first established that growth of CWR22Rv1 cells is inhibited by WIN-55,212-2 treatment (data not shown). Next, we determined the effect of WIN-55,212-2 treatment (5-30 μM) of CWR22Rv1 cells on the AR protein expression and found a significant decrease in its expression in a dose-dependent manner (Fig. 14). CWR22Rv1 cells exhibit high protein levels of intracellular PSA and WIN-55,212-2 (5-30 μM) treatment exhibited a significant decrease in PSA protein levels in dose-dependent manner (Fig.14).

0   5   10   15   20   25   30 μM **WIN-55,212-2**
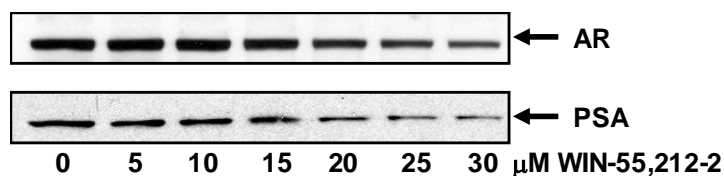
Fig. 14 Effect of WIN-55,212-2 on protein expression of AR and PSA in CWR22Rv1 cells. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μg protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the figures represent change as compared to vehicle treatment, in protein expression of the bands normalized to β-actin. Western blot data shown here are from a representative experiment repeated three times with similar results.

(15) Effect of WIN-55,212-2 on tumorigenecity of CWR22Rv1 cells in an athymic nude mice

Because WIN-55,212-2 was observed to be effective in inhibiting the growth of CWR22Rv1 cells *in vitro* and to establish *in vivo* relevance of our earlier published *in vitro* findings, we implanted athymic nude mice with CWR22Rv1 cells which form rapid tumors and secrete PSA in the blood stream of the host. WIN-55,212-2 treatment did not cause any loss in the body weight, food intake, or exhibited apparent signs of toxicity in animals. Implantation of CWR22Rv1 cells onto nude mice produced visible tumors in mice with a mean latent period of 11 days. Tumor growth, as inferred by computed tumor volume, was significantly inhibited in mice treated with WIN-55,212-2. In our experimental protocol, we euthanized the animals when the implanted tumor reached a volume of 1,200 mm^3. Thus, as shown in Fig. 15A & 15B, in control animals (group I) the average tumor volume of 1,200 mm^3 was reached in $\approx 35 \pm 3$ days after tumor cell inoculation. At this time point, the average tumor volume of the WIN-55,212-2 treated group II was 568 mm^3. In group III, we evaluated whether treatment of WIN-55,212-2 to animals leads to delay in the growth of tumors in nude mice. Tumor growth inhibitory response was observed in the group III, where the targeted average tumor volume of 1,200 mm^3 was reached at day 51 ± 4 after tumor cell inoculation (Fig. 15C). Tumor data were analyzed for survival probability by Kaplan-Meier analysis, which indicated that WIN-55,212-2 treatment to athymic nude mice resulted in increased survival, with a median survival of 51 days, compared with 35 days in control group.
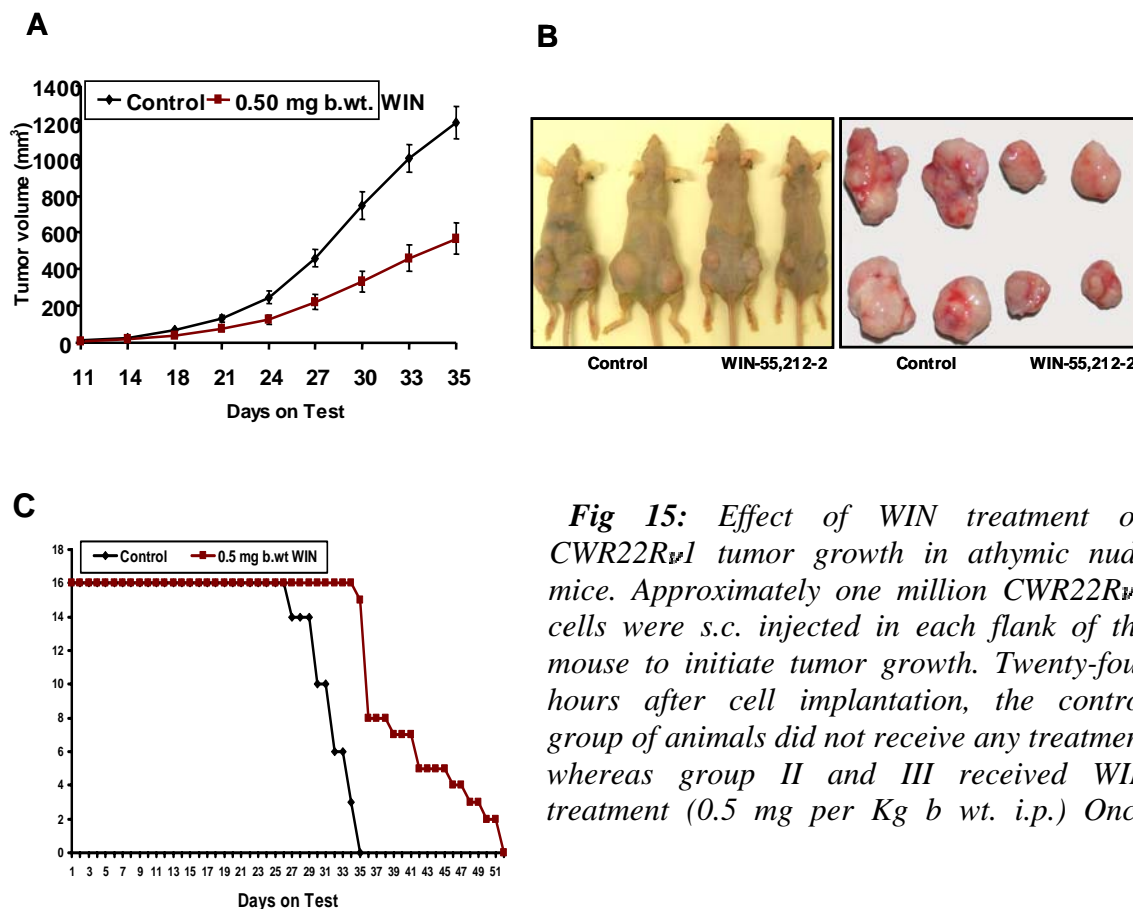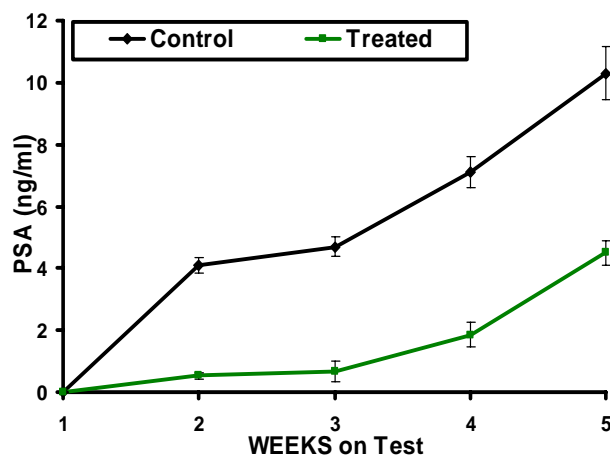

Fig 15: Effect of WIN treatment on CWR22Rv1 tumor growth in athymic nude mice. Approximately one million CWR22Rv1 cells were s.c. injected in each flank of the mouse to initiate tumor growth. Twenty-four hours after cell implantation, the control group of animals did not receive any treatment whereas group II and III received WIN treatment (0.5 mg per Kg b wt. i.p.) Once

tumors started to grow, their sizes were measured twice weekly and the tumor volume was calculated. (A) Average tumor volume of control and WIN treated mice was plotted over days after tumor cell inoculation. Values represent mean ± SD of eight animals. **, *P < 0.001 (B) Representative pictures of control mice and WIN treated mice on day (C) Tumor growth inhibitory response was observed in the group III, where the targeted average tumor volume of 1,200 mm³ was reached at day 51 ± 4 after tumor cell inoculation.*

(16) WIN-55,212-2 Inhibits PSA secretion in athymic nude mice implanted with CWR22Rv1 cells

Next, we determined the effect of WIN-55,212-2 on serum PSA levels in nude mice implanted with CWR22Rv1 tumors. During the course of tumor growth in animals, blood was collected through the mandibular bleed after every week. Quantitative sandwich ELISA was used to determine PSA levels in mouse serum secreted by CWR22Rv1 tumor xenografts. In WIN-55,212-2 treated animals a significant inhibition of PSA secretion was observed at all time points examined (Fig.16). At four weeks after inoculation, secreted PSA levels in control group was 7.1 ng/ml and 1.86 ng/ml in WIN-55,212-2 treated animals, respectively. At 35 days after inoculation of tumor cells in treated groups, 54 % ($P < 0.001$) reduced levels of PSA were observed as compared with the control group, (Fig.16). Thus, our data clearly demonstrated that WIN-55,212-2 treatment of mice resulted in a significant decrease in the serum PSA of animals. Interestingly, the effects of WIN-55,212-2 on PSA secretion were found to closely correlate with tumor growth inhibition (Fig.15 A, 15B).
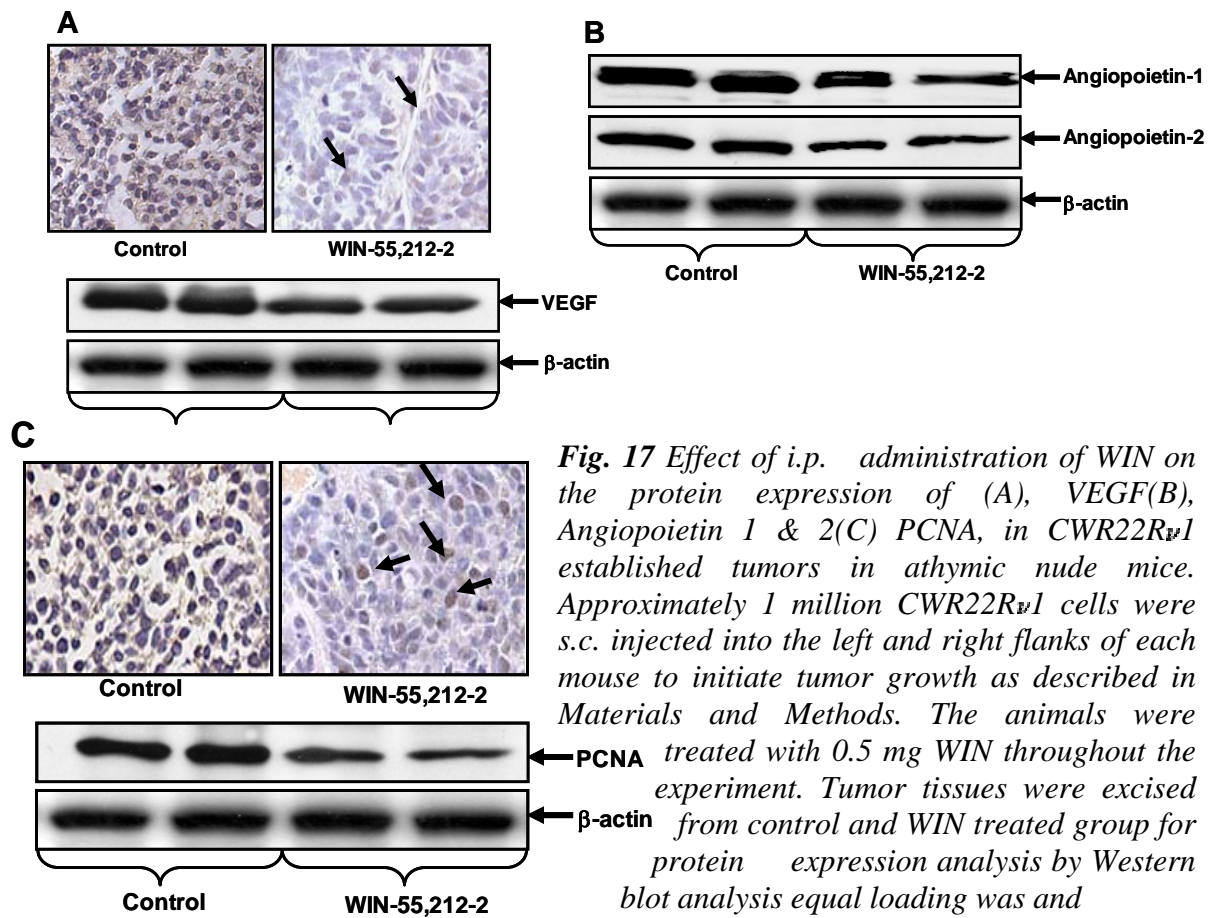

Fig 15: Effect of WIN treatment on CWR22Rv1 tumor growth in athymic nude mice. Approximately one million CWR22Rv1 cells were s.c. injected in each flank of the mouse to initiate tumor growth. Twenty-four hours after cell implantation, the control group of animals did not receive any treatment whereas group II received WIN treatment (0.5 mg per Kg b wt. i.p.) Once tumors started to grow, blood was collected once a week and serum PSA levels were analyzed by ELISA. Values represent mean ± SE of eight animals. ** *P < 0.001 vs. control group of mice. Details are described in Materials and Methods.*

(17) WIN-55,212-2 inhibits markers of angiogenesis VEGF, Angiopoietin 1 & 2 and PCNA in athymic nude mice implanted with CWR22Rv1 cells

Tumor tissues from control and treated mice were examined for biochemical variables at 35 days post-treatment when control tumors had reached a volume of 1,200 mm^3. At this time, tumor volume in the WIN treated group was 568 mm^3. Because our results suggested that WIN treatment induced tumor growth inhibition, we examined several molecules that have relevance to tumor growth. Our observations suggested decrease in the protein expression of vascular endothelial growth factor (VEGF), a marker for tissue angiogenesis (Fig 17A). Angiopoietins are protein growth factors that promote angiogenesis and angiopoietin 1 and 2 are known to be required for the formation of mature blood vessels. We observed a significant decrease in the protein expression of these proteins in tumors of mice treated with WIN (Fig. 17B). Further, protein expression of PCNA also significantly decreased in tumors of mice that received WIN treatment, compared with control tumors of mice (Fig. 17C).
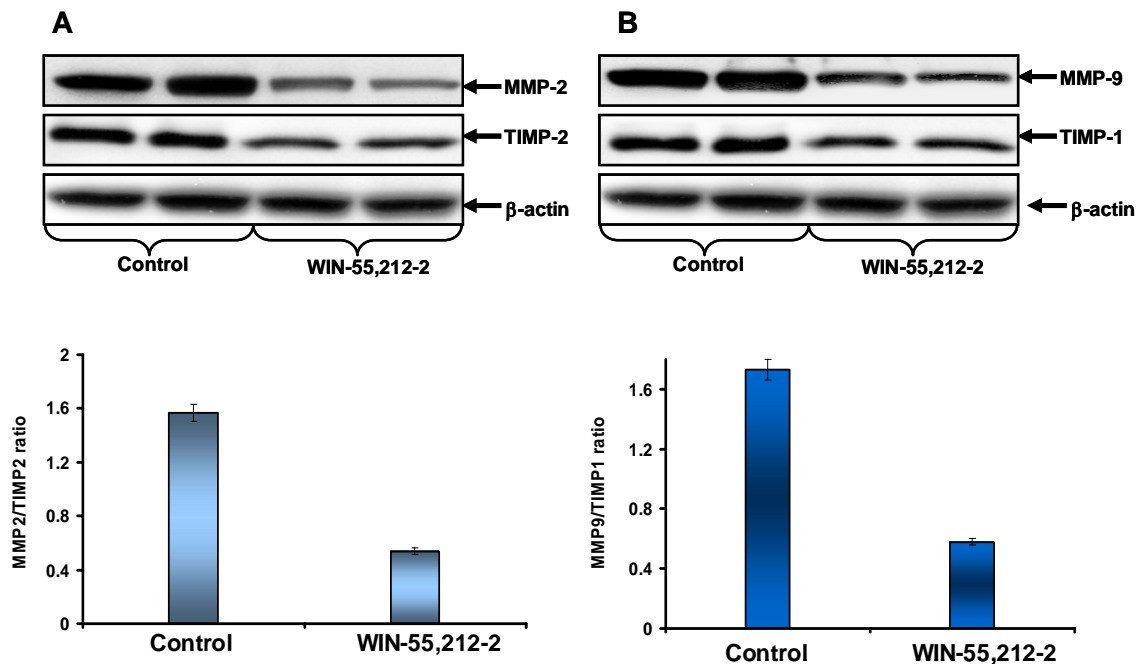

Fig. 17 *Effect of i.p. administration of WIN on the protein expression of (A), VEGF(B), Angiopoietin 1 & 2(C) PCNA, in CWR22Rv1 established tumors in athymic nude mice. Approximately 1 million CWR22Rv1 cells were s.c. injected into the left and right flanks of each mouse to initiate tumor growth as described in Materials and Methods. The animals were treated with 0.5 mg WIN throughout the experiment. Tumor tissues were excised from control and WIN treated group for protein expression analysis by Western blot analysis equal loading was and immunihistochemical analysis.*

(18) WIN-55,212-2 inhibits markers of metastasis in athymic nude mice implanted with CWR22Rv1 cells

There is considerable evidence that one class of metalloenzyme, the matrix metalloproteinase (MMP), plays a key role in matrix degradation, thus allowing tumor

dissemination. Prostate cancers with invasive potential are known to secrete active forms of MMP-2 and MMP-9 in a process that is inhibited by TIMP-1 and TIMP-2. In the next series of experiments, we investigated the tissue expression of MMPs and the TIMPs in control and WIN treated mice. We observed decrease in protein expression of MMP-2 and MMP-9 (Fig.18 A and B). Endogenous levels of tissue inhibitors of metalloproteinases (TIMP) specifically inhibit the matrix metalloproteinases. The expression of MMPs and TIMPs has been proposed to be coregulated, and an imbalance between them has been shown to be an essential factor in the invasive phenotype of cancers. Interestingly densitometric analysis of MMP to TIMP ratio in mice indicates that the balance was shifted in such a way that favored MMP expression in control mice, whereas WIN treatment of mice favored this ratio toward TIMP expression suggesting inhibition of MMP expression (Fig.18 A & 18 B).


Fig 18. *Effect of i.p. administration of 0.5 mg WIN on the protein expression of MMPs and TIMPs (A) protein levels of MMP-2 and TIMP-2 by immunoblot analyses and* MMP2/TIMP2 *ratio. (B) protein levels of MMP-9 and TIMP-1 by immunoblot analyses and MMP9/TIMP1 ratio. The ratios were determined from the data obtained by immunoblot analyses of MMPs and TIMPs.*

(19) Therapeutic potential of WIN-55,212-2 on tumorigenecity of CWR22Rν1 cells in an athymic nude mice

This protocol was designed to assess the therapeutic potential of WIN-55,212-2. For this purpose, CWR22Rν1 cells were inoculated onto the flanks of athymic nude mice as described under protocol 1. The animals were given autoclaved diet and water *ad libitum*

until the tumors were established to a volume of 400 mm^3. At this point, the animals were evenly divided into two groups and treatment was started. Control-cells were implanted (at the start of the experiment; Day 0), no further treatment was given; *Group II:* cells were implanted and tumors were allowed to grow to a volume of 400 mm^3, followed by i.p. injection of WIN-55,212-2 (0.5 mg b.wt.) three times a week. The treatment was continued until the tumor reached to a volume of 1200 mm^3. At this point, the animals were withdrawn from the study and euthanized. The time to reach an average tumor volume of 1200 mm^3 was 36 days post-inoculation in the control mice, 46 days for 0.5 mg WIN treated mice (Fig.19), a significant difference in tumor growth was observed in 0.5 mg WIN treated mice as compared with the controls ($P < 0.01$). Thus, our data confirmed the observations in the previous experiment and demonstrated a significant delay in tumor formation with physiologically achievable concentrations of WIN.
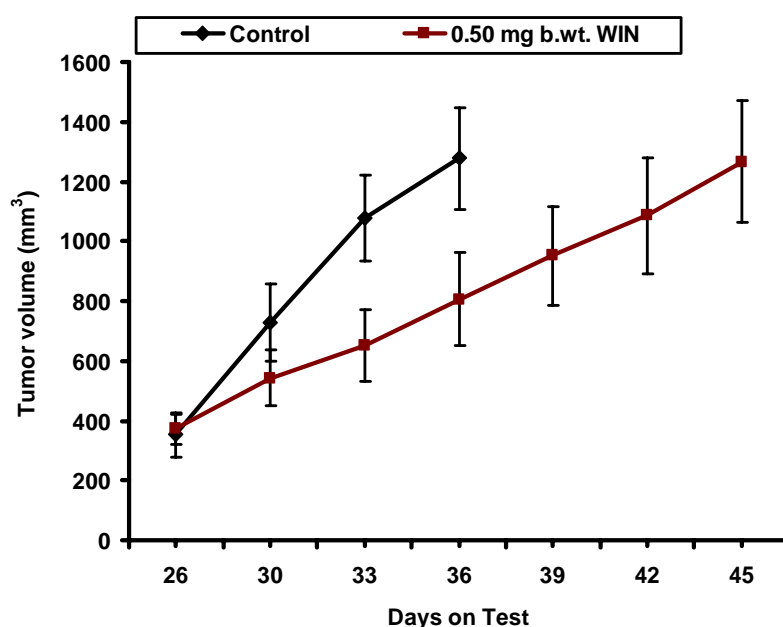
Fig 19. *Effect of WIN treatment on the growth of CWR22Rᵥ1 implanted tumors in athymic nude mice. CWR22Rᵥ1 cells (1 x 10^6) were implanted on the right and left flanks of athymic nude mice and the mice were treated with WIN as described in Materials and methods (protocol 2). The animals were followed for the development of tumors and the experiment terminated when the tumor volume reached 1200 mm^3.*

Key Research Accomplishments
    (i)   We found that expression levels of both cannabinoid receptors CB_1 and CB_2 are significantly higher in CA-HPV-10 (virally transformed cells, derived from adenocarcinoma of human prostate tissue), and other human prostate cells LNCaP, DUI45, PC3, and CWR22Rv1 than in human prostate epithelial

and PZ-HPV-7 (virally transformed cells, derived from normal human prostate tissue) cells.

(ii) We found that WIN-55,212-2 (mixed CB_1/CB_2 agonist) treatment to androgen-responsive LNCaP cells resulted in a dose (1-10 μM) and time-dependent (24-48 h) inhibition of cell growth.

(iii) We found that blocking of CB_1 and CB_2 receptors by their antagonists SR141716 (CB_1) and SR144528 (CB_2) significantly prevented the growth inhibitory effects exerted by WIN-55,212-2.

(iv) These data suggest that both CB_1 and CB_2 receptors may be involved in WIN-55,212-2 mediated growth inhibition and apoptosis.

We next conducted the study to understand the mechanistic basis of cannabinoid receptor-agonist induced prostate cancer cell growth inhibition and induction of apoptosis.

(v) Treatment of cells with WIN-55,212-2 was found to result in (a) an arrest of the cells in the G_0/G_1 phase of the cell cycle; (b) up regulation of ERK1/2, JNK1/2, p38; and (c) inhibition of PI3k/Akt pathways.

(vi) To define the involvement of regulatory proteins operative in the G_0/G_1 phase of the cell cycle, we determined the effects of WIN-55,212-2 treatment of cells on cyclin kinase inhibitor (cki)-cyclin-cyclin dependent kinase (cdk) machinery. We observed that WIN-55,212-2 (1-10 μM) treatment resulted in (a) an induction of p27/KIP1; (b) down-regulation of cyclin D1, D2, E; and (c) decrease in the expression of cdk -2, -4, and -6.

(vii) Western blot analysis showed a decrease in the protein expression of (a) pRb; (b) E2F (1 through 4); and (c) DP1 and DP2.

(viii) We found that WIN-55,212-2 induced apoptosis of human prostate cancer cells LNCaP proceeds through sustained activation of ERK1/2 leading to G_1 cell cycle arrest

(ix) WIN-55,212-2 treatment of cells resulted in a dose-dependent increase in Bax/Bcl-2 ratio in such a way that favors apoptosis. The induction of apoptosis proceeded through down regulation of caspases 3, 6, 7, and 9 and cleavage of PARP.

(x) WIN-55,212-2 treatment of CWR22Rν1 Cells inhibited androgen receptor and PSA protein expression.

(xi) WIN-55,212-2 treatment inhibits tumor growth and PSA levels in athymic nude mice implanted with CWR22Rν1 cells.

(xii) WIN-55,212-2 treatment inhibits markers of angiogenesis and PCNA in an athymic nude mice implanted with CWR22Rν1 cells

(xiii) WIN-55,212-2 treatment inhibits markers of metastasis in athymic nude mice implanted with CWR22Rν1 cells

(xiv) Our results suggest that WIN-55,212-2 or other non-habit forming cannabinoid receptor agonists could be developed as novel therapeutic agents for the treatment of prostate cancer.

Reportable Outcomes

Work described in this report was published in two major research articles. The first paper was published in **"Cancer Research"** where it featured as a **PRIORITY REPORT** in the March 1, (65): 5, 1635-1641, 2005 issue (appendix 1). The second paper was published in **"The Journal of Biological Chemistry"** Dec 22, (281) 51, 39480-39491, 2006 (appendix 2). In addition two abstract were presented as posters at the Annual meeting of "American Association of Cancer Research" held in Anaheim, CA from 16th to 20th April 2005 (appendix 3) and Washington D.C from 1st to 5th April 2006 (appendix 4). We have also published a review "Cannabinoids for Cancer Treatment: Progress and Promise in **"Cancer Research"** in the Jan 15, (68): 2, 339-342, 2008 issue (appendix 5).

Conclusions

It is now well accepted that uncontrolled cellular growth, which may be a result of defects in cell cycle and apoptotic machinery, is responsible for the development of most of the cancers including prostate cancer. Thus the agents which can modulate apoptosis in cancer cells may be able to affect the steady-state cell population and be useful in the management and therapy of cancer. Consistent with this notion, there is a need to develop novel targets and mechanism-based agents for the management of prostate cancer. One of the most exciting and promising areas of current cannabinoid research is the ability of these compounds to control the cell survival/death decision Our results suggest that treatment of androgen responsive human prostate carcinoma LNCaP cells resulted in a decrease in intracellular and secreted levels of PSA, with concomitant inhibition of AR, cell growth and induction of apoptosis. Based on the outcome of our mechanistic study of cannabinoid action and the available literature knowledge, and as shown in the composite scheme in Fig 20, we suggest that cannabinoid receptor agonist WIN-55,212-2 induces sustained and prolong activation of ERK1/2, which leads to induction of cyclin kinase inhibitor p27/KIP1, which inturn inhibits cell cycle regulatory molecules resulting in G_1 arrest and apoptosis. Downregulation of cdk4/6 inhibits pRb which inhibits protein expression of E2F family of proteins and its heterodimeric partners DP1 and DP2 leading to gene transcription and apoptosis. Since, Bax and Bcl-2 play a critical role in induction of apoptosis, alteration of Bax/Bcl-2 ratio activates caspase signaling resulting in apoptotic cell death. To establish *in vivo* relevance of these *in vitro* findings, we implanted athymic nude mice with androgen-responsive CWR22Rv1 cells which form rapid tumors and secrete PSA in the blood stream of the host. As compared to untreated animals, WIN treated mice (0.5 mg/kg b.wt, i.p, alternate day) exhibited significant inhibition in the tumor growth with significant reduction in PSA secretion in the serum. In animals without WIN treatment, targeted tumor volume of 1200 mm^3 was reached at 35 days post-tumor inoculation; whereas this tumor volume was attained in 51 days in WIN treated mice. Hence, we conclude that cannabinoids receptor agonist should be

considered as effective agents for the treatment of prostate cancer and the long term implications of our study could be to develop non-habit forming cannabinoid agonist (s) for the management of prostate cancer.
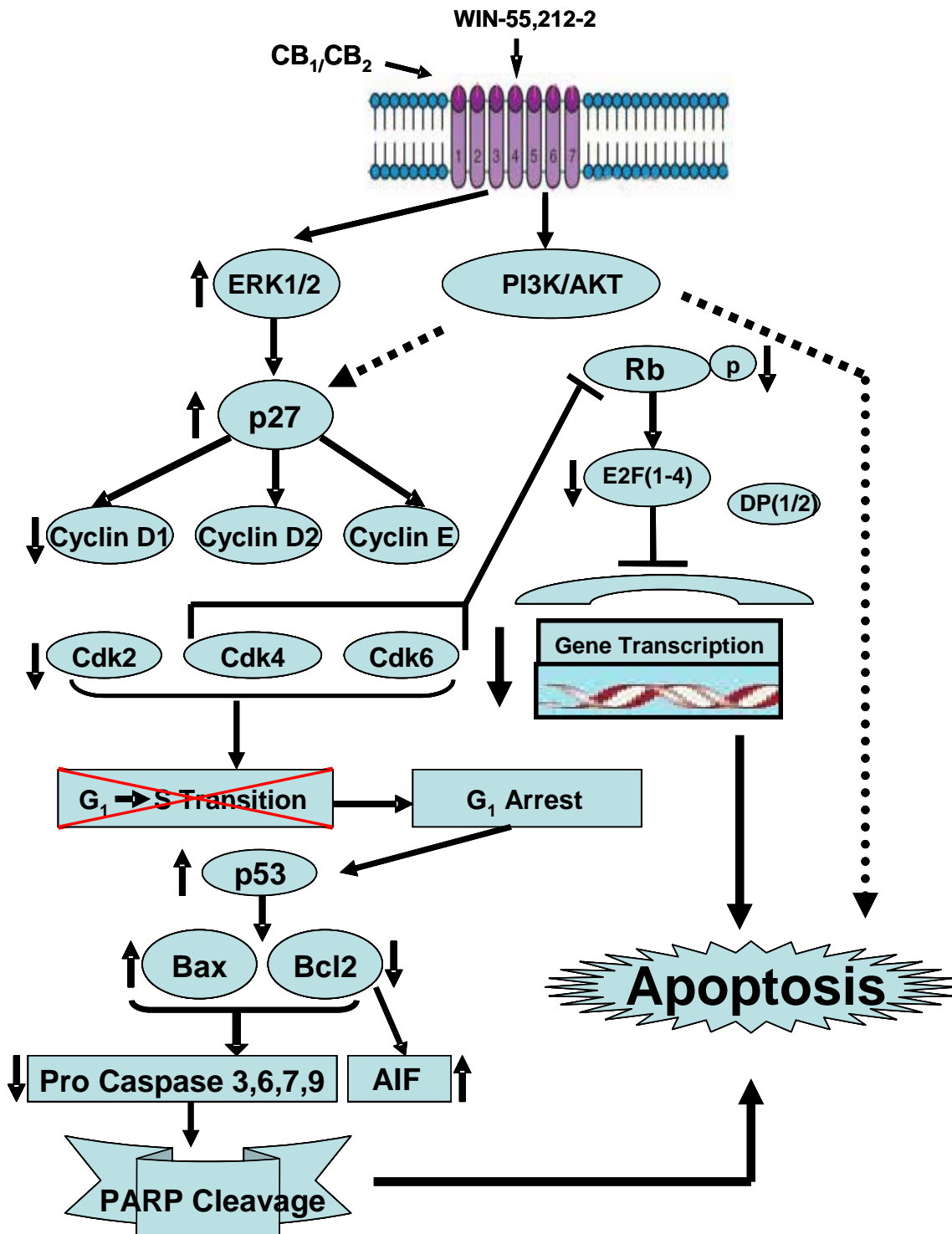
Fig 20: *Proposed schematic model for WIN-55,212-2-mediated cell cycle dysregulation and induction of apoptosis.*

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Appendix 1

Cannabinoid Receptor as a Novel Target for the Treatment of Prostate Cancer

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Abstract

Cannabinoids, the active components of *Cannabis sativa* Linnaeus (marijuana) and their derivatives have received renewed interest in recent years due to their diverse pharmacologic activities such as cell growth inhibition, anti-inflammatory effects and tumor regression. Here we show that expression levels of both cannabinoid receptors, CB_1 and CB_2, are significantly higher in CA-human papillomavirus-10 (virally transformed cells derived from adenocarcinoma of human prostate tissue), and other human prostate cells LNCaP, DUI45, PC3, and CWR22Rν1 than in human prostate epithelial and PZ-HPV-7 (virally transformed cells derived from normal human prostate tissue) cells. WIN-55,212-2 (mixed CB_1/CB_2 agonist) treatment with androgen-responsive LNCaP cells resulted in a dose- (1-10 μmol/L) and time-dependent (24-48 hours) inhibition of cell growth, blocking of CB_1 and CB_2 receptors by their antagonists SR141716 (CB_1) and SR144528 (CB_2) significantly prevented this effect. Extending this observation, we found that WIN-55,212-2 treatment with LNCaP resulted in a dose- (1-10 μmol/L) and time-dependent (24-72 hours) induction of apoptosis (*a*), decrease in protein and mRNA expression of androgen receptor (*b*), decrease in intracellular protein and mRNA expression of prostate-specific antigen (*c*), decrease in secreted prostate-specific antigen levels (*d*), and decrease in protein expression of proliferation cell nuclear antigen and vascular endothelial growth factor (*e*). Our results suggest that WIN-55,212-2 or other non–habit-forming cannabinoid receptor agonists could be developed as novel therapeutic agents for the treatment of prostate cancer. (Cancer Res 2005; 65(5): 1635-41)

Introduction

Because prostate cancer has become the most common cancer diagnosed in men, developing novel targets and mechanism-based agents for its treatment has become a challenging issue. In recent years cannabinoids, the active components of *Cannabis sativa* Linnaeus (marijuana) and their derivatives have drawn renewed attention because of their diverse pharmacologic activities such as cell growth inhibition, anti-inflammatory effects, and tumor regression (1–5). Cannabinoids have been shown to induce apoptosis in gliomas (6), PC-12 pheochromocytoma (7), CHP 100 neuroblastoma (8), and hippocampal neurons (9) *in vitro*, and most interestingly, regression of C6-cell gliomas *in vivo* (10). Further interest in cannabinoid research came from the discovery

of specific cannabinoid systems and the cloning of specific cannabinoid receptors (10). These diversified effects of cannabinoids are now known to be mediated by the activation of specific G protein-coupled receptors that are normally bound by a family of endogenous ligands, the endocannabinoids (11, 12). Two different cannabinoid receptors have been characterized and cloned from mammalian tissues: the "central" CB_1 receptor (13), and the "peripheral" CB_2 receptor (14).

In the present study, we show for the first time that expression levels of both cannabinoid receptors, CB_1 and CB_2, are higher in human prostate cancer cells than in normal cells. Importantly, we also show that WIN-55,212-2 (CB_1/CB_2 agonist) treatment with androgen-responsive LNCaP cells results in a dose- and time-dependent inhibition of cell growth with a concomitant induction of apoptosis, decrease in protein and mRNA expression of androgen receptor and prostate-specific antigen (PSA), decrease in secreted PSA levels, protein expression of proliferating cell nuclear antigen (PCNA), and vascular endothelial growth factor (VEGF). We suggest that cannabinoid receptor agonists may be useful in the treatment of human prostate cancer.

Materials and Methods

Materials. *R*-(+)-WIN-55,212-2 (2,3 dihydro-5-methyl-3 [(morpholinyl)methyl]pyrollo (1,2,3 de)-1,4-benzoxazinyl]-[1-naphthaleny]methanone, $C_{27}H_{26}N_2O_3 \cdot CH_3SO_3H$ was purchased from Sigma (St. Louis, MO). CB_1 receptor antagonist SR141716 (SR1) and CB_2 receptor antagonist SR144528 (SR2) were procured from Dr. Herbert H. Seltzman (National Institute on Drug Abuse, Division of Neuroscience and Behavioral Research, through RTI International, Research Triangle Park, NC). DMEM and fetal bovine serum were procured from Life Technologies, Invitrogen Corporation (Grand Island, NY). Human PSA ELISA kit from Yes Biotech Laboratories (Ontario, Canada) and annexin-V-FLUOS staining kit from Roche Diagnostic Corporation (Indianapolis, IN). Antibiotics (penicillin and streptomycin) used were obtained from Cellgro Mediatech, Inc. (Herndon, VA). Apo-Direct kit for measuring apoptosis by flow cytometry was procured from Apo-Direct (San Diego, CA). RNA isolation kit was from Qiagen, Inc. (Valencia, CA). Monoclonal antibodies for PSA, androgen receptor, PCNA, and VEGF, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse secondary horseradish peroxidase conjugate was obtained from Amersham Biosciences Limited (Buckinghamshire, United Kingdom). Protein was estimated using bicinchoninic acid protein assay kit obtained from Pierce (Rockford, IL).

Cell Culture. The LNCaP, DU145, PC-3, CWR22Rν1, CA-HPV-10, and PZ-HPV-7 cells were obtained from American Type Culture Collection (Manassas, VA). LNCaP and DU145 cells were cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. PC-3 and CWR22Rν1 cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. CA-HPV-10 and PZ-HPV-7 were grown in keratinocyte serum-free medium (17005-042, Life Technologies) supplemented with 5 ng/mL human recombinant EGF and 50 μg/mL bovine pituitary extract. Human prostate epithelial cells (PrEC) were obtained from Cambrex Bioscience (Walkersville, MD) and grown in prostate epithelial basal cell medium (Cambrex Bioscience)

according to the manufacturer's instructions. The cells were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment.

Treatment of Cells. WIN-55,212-2 (dissolved in DMSO), was used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies, cells were treated with WIN-55,212-2 at final concentrations of 1.0, 2.5, 5.0, 7.5, and 10.0 μmol/L for 24 hours in complete cell medium, whereas for time-dependent studies, the cells (50-60% confluent) were treated with 5 μmol/L dose of WIN-55,212-2 for 24, 48, and 72 hours. For time-dependent study, we included a control treated with DMSO for 72 hours because it was the longest time point post-WIN-55,212-2 treatment in our experimental protocol. To establish the role of CB_1 and CB_2 receptors in WIN-55,212-2–induced inhibitory effects, two experiments were done. In the first experiment, cells were treated with 2 μmol/L of SR141716 or SR144528 alone for 24 hours. In the second experiment, cells pretreated with each of these antagonists (2 μmol/L) for 3 hours followed by incubation with 7.5 μmol/L WIN-55,212-2 for 24 hours. In pilot experiments, it was established that DMSO (0.1% v/v) had no effects when measured at 24, 48, or 72 hours.

Cell Viability. The effect of WIN-55,212-2 on the viability of cells was determined by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) (MTT) assay. The cells were plated at 1×10^4 cells per well in 200 μL of complete culture medium containing 1.0, 2.5, 5.0, 7.5, and 10.0 μmol/L concentrations of WIN-55,212-2 in 96-well microtiter plates for 24 and 48 hours at 37°C in a humidified chamber. Each concentration of WIN-55,212-2 was repeated in 10 wells. After incubation for specified times at 37°C in a humidified incubator, MTT reagent (4 μL, 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4°C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 μL). Absorbance was recorded on a microplate reader at 540 nm wavelength. The effect of WIN-55,212-2 on growth inhibition was assessed as the percentage of inhibition in cell growth where vehicle-treated cells were taken as 100% viable.

Preparation of Cell Lysates and Western Blot Analysis. Following treatment of cells with WIN-55,212-2, the medium was aspirated and the cells were washed with cold PBS [10 mmol/L (pH 7.45)]. The cells were then incubated in ice-cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)], with freshly added protease inhibitor cocktail (protease inhibitor cocktail set III, Calbiochem, La Jolla, CA) over ice for 20 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21.5-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at $14,000 \times g$ for 15 minutes at 4°C, and the supernatant (total cell lysate) was collected, aliquoted, and used on the day of preparation or immediately stored at −80°C for use at a later time. For Western blotting, 25 to 50 μg of protein were resolved over 12% polyacrylamide gels and transferred onto a nitrocellulose membrane. The nonspecific sites on blots were blocked by incubating in blocking buffer [5% nonfat dry milk/1% Tween 20 in 20 mmol/L TBS (pH 7.6)] for 1 hour at room temperature, incubated with appropriate monoclonal primary antibody in blocking buffer for 90 minutes to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate and detected by chemiluminescence and autoradiography using Hyperfilm obtained from Amersham Biosciences. Densitometric measurements of the bands in Western blot analysis were done using digitalized scientific software program UN-SCAN-IT purchased from Silk Scientific Corporation (Orem, UT).

ELISA for PSA. The human PSA ELISA kit was used for the quantitative determination of PSA levels in culture medium according to the vendor's protocol. This kit uses a technique of quantitative sandwich immunoassay for determination of PSA with an estimated sensitivity of 1 ng/mL PSA antigen.

Detection of Apoptosis and Necrosis by Confocal Microscopy. The annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells according to vendor's protocol. This kit uses a dual-staining protocol in which the apoptotic cells are stained with annexin-V (green fluorescence), and the necrotic cells are stained with propidium iodide (PI; red fluorescence). LNCaP cells were grown to about 70% confluence and then treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, and 10.0 μmol/L) for 24 hours. The fluorescence was measured by a Zeiss 410 confocal microscope (Thornwood, NY). Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514 to 540 nm bandpass barrier filter. Images of red PI fluorescence were collected using a 568 nm excitation light from the argon/krypton laser, a 560 nm dichroic mirror, and a 590-nm-long pass filter. In a selected field, the cells stained with annexin-V and PI as well as unstained cells were counted to ascertain the extent of apoptosis and necrosis.

Quantification of Apoptosis by Flow Cytometry. The cells were grown at density of 1×10^6 cells in 100 mm culture dishes and were treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, and 10.0 μmol/L doses) for 24 hours. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged dUTP nucleotide and PI by use of an Apo-Direct apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) and was used according to the manufacturer's protocol. The labeled cells were analyzed by flow cytometry.

Quantitative Real-time PCR for mRNA Expression of Androgen Receptor and PSA. Total RNA was isolated from LNCaP cells using RNeasy kit according to the vendor's protocol. The ratio of optical densities of RNA samples at 260 and 280 nm was consistently >1.8. cDNA was synthesized by reverse transcription of 1 μg of extracted RNA with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in the presence of oligo dT and deoxynucleotide triphosphate (Promega). Androgen receptor and PSA were amplified using a Failsafe real-time PCR system obtained from Epicentre (Madison, WI). The thermal cycler used for amplification was an ABI-PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers were designed as follows: androgen receptor forward, 5′-AAGACGCTTCTACCAGCTCAC-CAA; reverse, 5′-TCCCAGAAAGGATCTTGGGCACTT; PSA forward, 5′-ACTCACAGCAAGGATGGAGCTGAA; reverse, 5′-TGAGGGTTGTCTG-GAGGACTTCAA. The cycler was programmed with the following conditions (a) initial denaturation at 94°C for 2 minutes, followed by 35 cycles of (b) 94°C for 40 seconds, (c) annealing of the primer template at 58°C for 40 seconds, and (d) extension at 72°C for 40 seconds.

Results

Expression of Cannabinoid Receptor in Normal and Prostate Cancer Cells. We first compared the expression levels of both cannabinoid receptors CB_1 and CB_2 in PrEC and a series of human prostate cancer cells. We also included a pair of cells, PZ-HPV-7 (virally transformed cells, derived from normal human prostate tissue) and CA-HPV-10 (virally transformed cells, derived from the adenocarcinoma of human prostate tissue) derived from the same individual. Immunoblot data shown in Fig. 1 revealed that expression of both CB_1 and CB_2 receptors was significantly higher in prostate cancer cells LNCaP, DUI45, PC3, CWR22Rν1, and CA-HPV-10 as compared with normal prostate cells PZ-HPV-7 and PrEC cells. To establish the specificity of the cannabinoid receptor antibodies used in the blotting experiments, antigen preabsorption experiments were carried out. The peptides blocked anti-CB_1 and anti-CB_2 antibody binding in all cells (data not shown).

Effect of WIN-55,212-2 on Cell Viability of PrEC and LNCaP Cells. To evaluate the cell viability response of WIN-55,212-2 on PrEC and LNCaP cells, MTT assay was employed. Data in Fig. 2A shows that treatment of PrEC cells with WIN-55,212-2 (1-10 μmol/L) for 24 and 48 hours had no effect on cell viability (Fig. 2A). However,

Figure 1. Western blot analysis of CB$_1$ and CB$_2$ cannabinoid receptor expression in normal and human prostate cancer cells. *A*, a pair of normal (PZ-HPV-7) and prostate cancer cells (CA-HPV-10) obtained from the same individual; *B*, PrEC (normal prostate epithelial cells) and prostate cancer cells, LNCaP, DU145, PC-3, and CWR22Rv1. Total cell lysates were prepared and 30 μg of protein were subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. β-Actin was used as a loading control. For other details, see Materials and Methods.
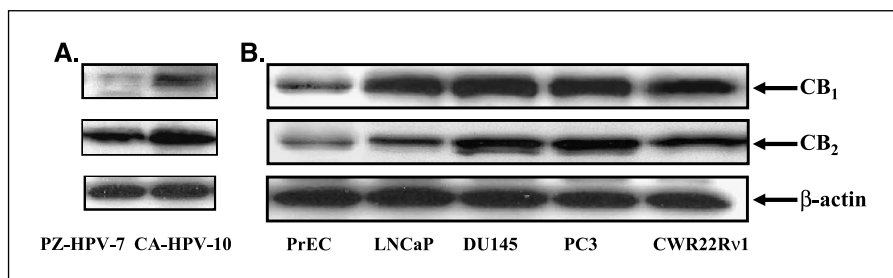
treatment of LNCaP cells with similar doses of WIN-55,212-2 in a dose-dependent manner significantly decreased the viability of LNCaP cells at 24 and 48 hours (Fig. 2*A*). The IC$_{50}$ for inhibition of cell viability at 24 and 48 hours was 6.0 and 5.0 μmol/L, respectively.

CB$_1$ and CB$_2$ receptor activation signals growth inhibition in LNCaP cells. To study the possible implication of CB$_1$ and CB$_2$ receptors in WIN-55,212-2–induced cell death, the effect of their antagonists were evaluated using MTT assay. Cells pretreated with 2 μmol/L of SR141716 (CB$_1$ antagonist) or SR144528 (CB$_2$ antagonist) had no effect on cell viability but exhibited significant protective effect when coadministered with WIN-55,212-2 (7.5 μmol/L) at a molar ratio of 1:3.75 (Fig. 2*B*). These data suggest that both CB$_1$ and CB$_2$ receptors may be involved in WIN-55,212-2–mediated growth inhibition and apoptosis.
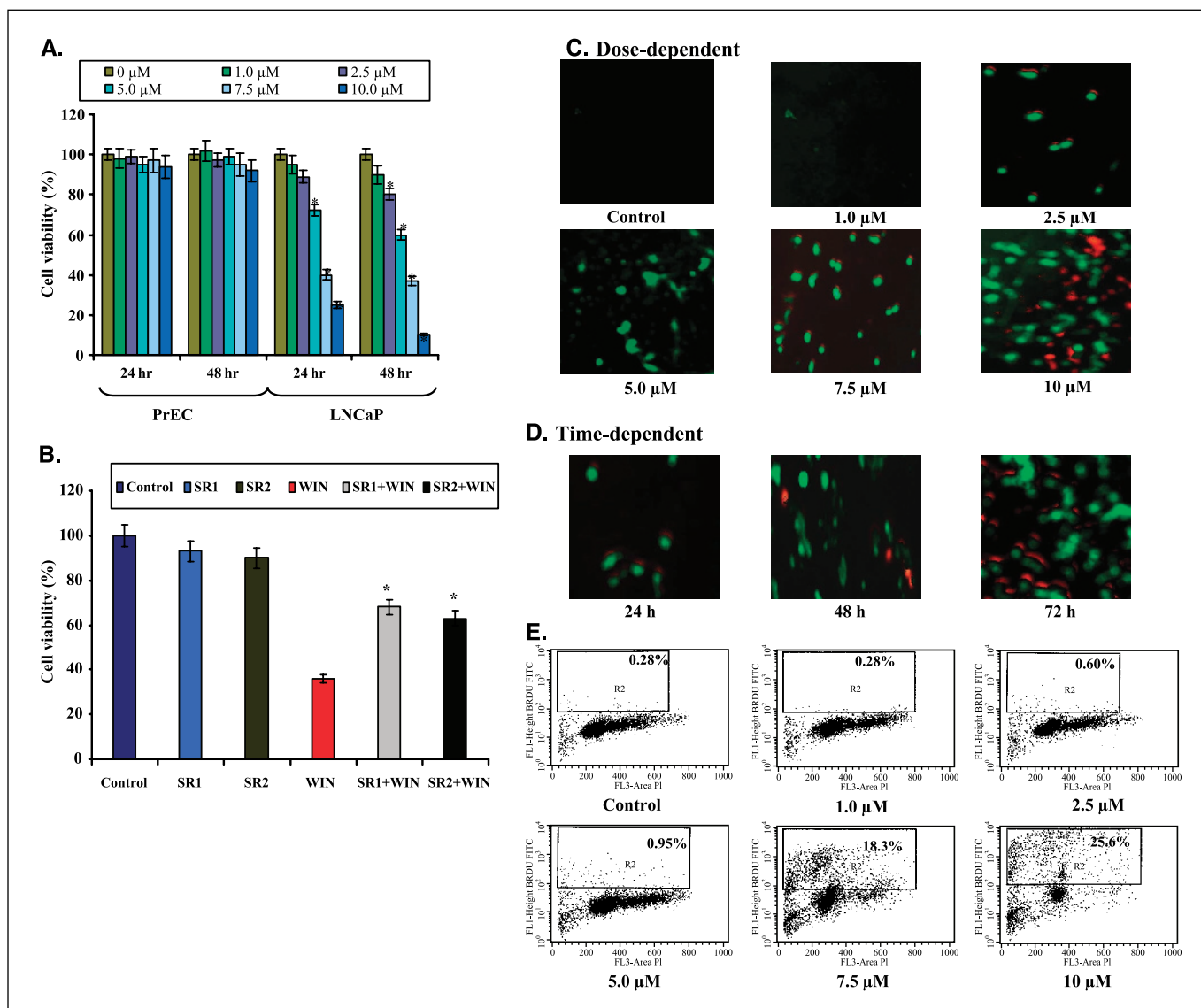
Effect of WIN-55,212-2 on Apoptosis and Necrosis of LNCaP Cells. We next assessed whether the cell growth inhibitory effect of WIN-55,212-2 was associated with induction of apoptosis. The induction of apoptosis by WIN-55,212-2 was evident from the analysis of the data obtained by confocal microscopy after labeling the cells with annexin-V (Fig. 2*B*). This method was used because it identifies the apoptotic (green fluorescence) as well as necrotic (red fluorescence) cells. As shown by the data, WIN-55,212-2 treatment resulted in a dose-dependent apoptosis in LNCaP cells. In a time-dependent study with a 5 μmol/L dose of WIN-55,212-2, there was an increasing trend of apoptotic cells at 72 compared with 48 hours after treatment (Fig. 2*C*). We next quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with dUTP and PI. LNCaP cells were treated with of WIN-55,212-2 (1-10 μmol/L) for 24 hours. As shown by the data in Fig. 2*D*, WIN-55,212-2 treatment resulted in 18.3% and 25.6% of apoptotic cells at a dose of 7.5 and 10 μmol/L, respectively. Whereas the induction of apoptosis was almost negligible at the lowest dose (1.0 μmol/L) used, the highest dose employed (10 μmol/L) resulted in a massive induction of apoptosis as determined by flow cytometry. A similar trend was evident when apoptosis was measured by ladder formation on agarose gel electrophoresis (data not shown).

Effect of WIN-55,212-2 on Androgen Receptor and PSA Protein and mRNA Expression in LNCaP Cells. Androgens are involved in the development and progression of prostate cancer where androgen receptor is assumed to be the essential mediator for androgen action (15, 16). In the next series of experiments, we determined the effect of WIN-55,212-2 on protein and mRNA expression of androgen receptor. In a dose-dependent study, we found that treatment of LNCaP cells with WIN-55,212-2 resulted in a marked decrease in androgen receptor protein expression (Fig. 3*A*). Relative density data of these immunoblots revealed that the decrease in androgen receptor protein expression was 50% and 90% at 5.0 and 7.5 μmol/L of WIN-55,212-2, respectively.

In a time-dependent study with 5 μmol/L dose of WIN-55,212-2, there was a marked decrease in androgen receptor protein expression and this corresponded with the relative density data showing a decrease of 61% and 69% at 48 and 72 hours, respectively (Fig. 3*B*). Studies have also shown that modulation in androgen receptor leads to alteration in androgen-responsive genes (17). PSA is an androgen-responsive gene and is regarded as the most sensitive biomarker and screening tool for prostate cancer in humans (18). The dose-dependent effect of WIN-55,212-2 on LNCaP cells showed a significant decrease in PSA protein expression at 5.0, 7.5, and 10 μmol/L concentrations when assessing at 24 hours post-treatment (Fig. 3*A*). Densitometric analysis data revealed that the decrease was 48%, 75%, and 90% at 5.0, 7.5, and 10.0 μmol/L concentrations (Fig. 3*A*). For time-dependent studies, cells were treated with 5 μmol/L of WIN-55,212-2 for 24, 48, and 72 hours. Employing Western blot analysis, we found a significant decrease in a time-dependent manner in PSA protein expression. Densitometric analysis revealed a decrease in PSA protein expression by 48% and 60% at 48 and 72 hours, respectively (Fig. 3*B*). We also evaluated the effect of WIN-55,212-2 on mRNA levels of androgen receptor and PSA. As shown by the real time-PCR analysis data, there was an inhibition in mRNA levels of androgen receptor (Fig. 3*C*) and PSA (Fig. 3*D*) at 7.5 and 10.0 μmol/L concentrations.

We next examined the effect of WIN-55,212-2 on secreted levels of PSA in LNCaP cells. Employing ELISA technique, we found that treatment of LNCaP cells with WIN-55,212-2 resulted in a dose-dependent decrease in the secreted levels of PSA by 30%, 53%, and 62% at 5.0, 7.5, and 10 μmol/L, respectively. At similar doses of WIN-55,212-2, but varying the time point by 48 hours, PSA levels decreased by 53%, 77%, and 80% (Fig. 3*E*). Furthermore, at 72 hours post-treatment of WIN-55,212-2, secreted PSA levels decreased by 58%, 82%, and 88% (Fig. 3*E*). From these data, it seems that the decrease in LNCaP cell growth was concomitant with a decrease in androgen receptor protein expression as well as a decrease in both intracellular and secreted PSA levels.

Effect of WIN-55,212-2 on Cell Proliferation Marker, PCNA. We next determined the effect of WIN-55,212-2 on PCNA which serves as a requisite auxiliary protein for DNA polymerase δ-driven DNA synthesis and is cell-regulated (19, 20). The dose-dependent study treatment of LNCaP cells with WIN-55,212-2 (1-10 μmol/L) resulted in a significant decrease in protein expression of PCNA. Western blot analysis and relative density of these bands showed that the decrease in protein expression of PCNA was 71% at 7.5 μmol/L WIN-55,212-2 (Fig. 4*A*). In a time-dependent study, treatment of LNCaP cells with 5 μmol/L WIN-55,212-2 resulted in >50% inhibition in PCNA protein expression at 48 and 72 hours of treatment (Fig. 4*B*).

Figure 2. Effect of WIN-55,212-2 on cell viability and apoptosis in LNCaP cells. *A,* effects on the viability of PrEC and LNCaP cells. As detailed in Materials and Methods, the cells were treated with WIN-55,212-2 (1-10 μmol/L) for 24 and 48 hours, and their viability was determined by MTT assay. *Columns,* means; *bars,* ± SE of three separate experiments in which each treatment was done in 10 wells; *, $P < 0.001$ compared with control (0 μmol/L). *B,* effects of CB_1 receptor antagonist SR141716 and CB_2 receptor antagonist SR144528 on WIN-55,212-2–induced cell viability. As detailed in Materials and Methods, cells were treated with 2 μmol/L of SR141716 or SR144528 alone for 24 hours. In another parallel set, cells were pretreated with each of these antagonists for 3 hours, followed by incubation with 7.5 μmol/L WIN-55,212-2 for 24 hours and their viability was determined by MTT assay. *Columns,* means; *bars,* ± SE of three separate experiments in which each treatment was done in 10 wells; *, $P < 0.001$ compared with WIN. *C* and *D,* induction of apoptosis by confocal microscopy, cells were treated with WIN-55,212-2 (1-10 μmol/L) for 24 hours for the dose-dependent study (*C*), and WIN-55,212-2 (5 μmol/L) for 24, 48, and 72 hours for the time-dependent study (*D*). The annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells. This kit uses a dual-staining protocol in which apoptotic cells are stained with annexin-V (*green fluorescence*) and necrotic cells are stained with PI (*red fluorescence*). *E,* quantification of apoptosis by flow cytometry. The cells were treated with WIN-55, 212-2 (1-10 μmol/L) for 24 hours, labeled with dUTP using terminal deoxynucleotide transferase and PI. Cells showing fluorescence (R2) are considered as apoptotic cells and their percentage population is indicated. Data from representative experiments repeated thrice with similar results.

Effect of WIN-55,212-2 on VEGF. Because VEGF is a marker for angiogenesis, blocking the angiogenic process may represent a promising way of treating the tumor. Studies have shown that androgen regulates VEGF content in prostate cancer (21). As WIN-55,212-2 treatment resulted in a decrease in androgen receptor expression, the effects on VEGF were also determined. It was observed that WIN-55,212-2 treatment also resulted in a decrease in VEGF protein expression (Fig. 4*A*). Densitometric analysis data showed a decrease of 40% at 7.5 μmol/L concentration of WIN-55,212-2. In a time-dependent study at 5 μmol/L WIN-55,212-2 treatment, VEGF protein expression decreased in a time-dependent manner (Fig. 4*B*).

Discussion

It is now well accepted that uncontrolled cellular growth, which may be a result of defects in cell cycle and apoptotic machinery, is responsible for the development of most of the cancers including prostate cancer. Thus, the agents which can modulate apoptosis in cancer cells may be able to affect the

steady-state cell population and may be useful in the management and therapy of cancer. Consistent with this notion, there is a need to develop novel targets and mechanism-based agents for the management of prostate cancer. One of the most exciting and promising areas of current cannabinoid research is the ability of these compounds to control the cell survival/death decision (1). In this study, we found that compared with PrEC and PZ-HPV-7 cells, the expression levels of both cannabinoid receptors CB_1 and CB_2 were significantly higher in CA-HPV-10 and other human prostate cells LNCaP, DU145, PC-3, and CWR22Rν1. These data suggest that CB_1 and CB_2 receptors could be a target for novel treatment options for prostate cancer. We also found that mixed CB_1/CB_2 agonist WIN-55,212-2 treatment of LNCaP cells resulted in a decrease of cell viability as determined by MTT assay at varying doses and time points (Fig. 2*A*), suggesting the involvement of both CB_1 and CB_2 in the antiproliferative action

of cannabinoids (Fig. 2*B*). It is widely recognized that apoptosis is an ideal way of elimination of cancer cells and that selective apoptotic events could provide suitable targets for cancer treatment and prevention. In this study, we also observed an increase in apoptosis of LNCaP cells by treatment with WIN-55,212-2. This observation was confirmed by employing confocal microscopy (Fig. 2*C* and *D*) and flow cytometry (Fig. 2*E*). This could be an important observation which might be useful for devising strategies for the management of human prostate cancer because apoptosis is a physiological and discrete way of cell death different from necrotic cell death and is regarded to be an ideal way of cell elimination.

Androgens are essential for the growth, differentiation, and functioning of the prostate as well as in increasing prostate cancer development (22, 23). Many molecular mechanisms have been suggested for the development of recurrent hormone refractory
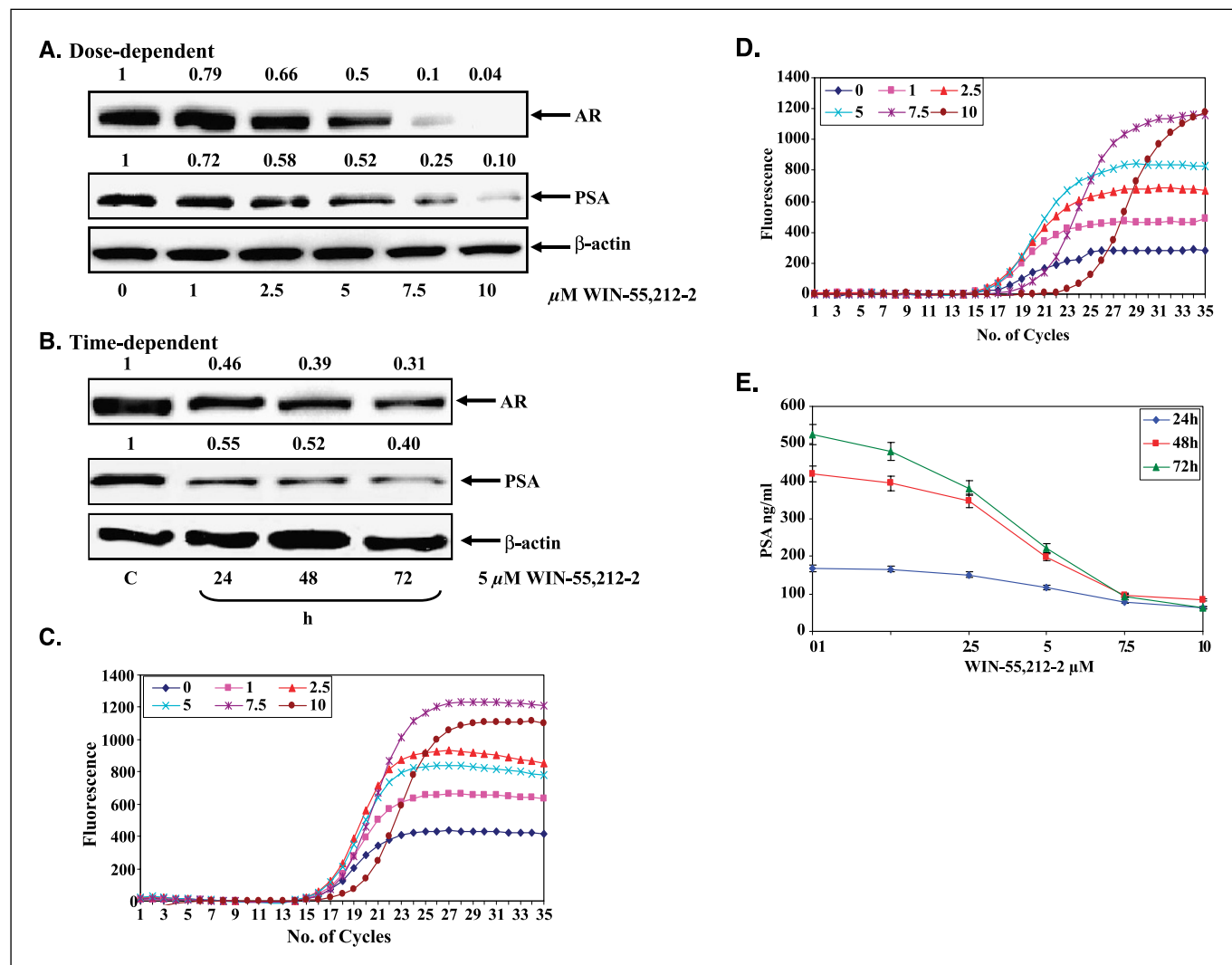
Figure 3. Effect of WIN-55,212-2 on protein and mRNA expression of androgen receptor and PSA in LNCaP cells. *A,* dose-dependent effect; and *B,* time-dependent effect. As detailed in Materials and Methods, the cells were treated with DMSO alone or with specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μg of protein were subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the blots represent change as compared with vehicle treatment in protein expression of the bands normalized to β-actin. Western blot data from a representative experiment repeated thrice with similar results. *C* and *D*, effects of WIN-55,212-2 on mRNA expression of androgen receptor (*C*) and PSA (*D*) determined by real time-PCR from representative experiments repeated twice with similar results. *E,* effect on secreted levels of PSA. Cells were treated with WIN-55,212-2 (1-10 μmol/L) for 24, 48, and 72 hours and then harvested. The PSA levels were determined by ELISA as described under Materials and Methods. *Points,* means; *bars,* ± SE of three independent experiments.
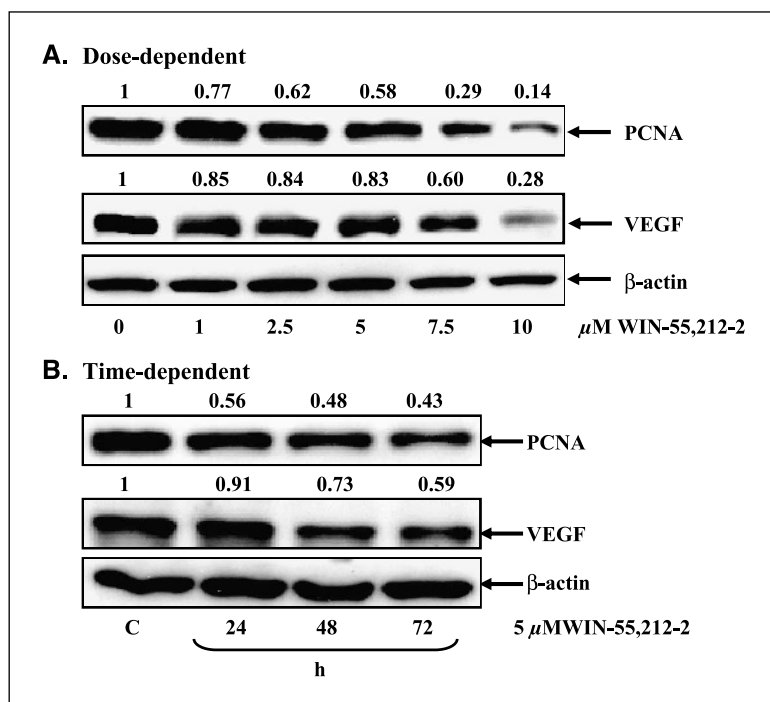
Figure 4. Effect of WIN-55,212-2 on protein expression of PCNA and VEGF in LNCaP cells. *A*, dose-dependent effect; *B*, time-dependent effect. As detailed in Materials and Methods, the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 µg of proteins were subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the blots represent change as compared with vehicle treatment in protein expression of the bands normalized to β-actin. Data from a representative experiment repeated thrice with similar results.

tumors. Most of these mechanisms postulate an alteration in the function of the androgen receptor and its signaling pathways (23). The overexpression of androgen receptor in prostate cancer may promote cell growth. Hence, elimination or reducing the androgen receptor in prostate cancer should help in treating this neoplastic disease. As most of the molecular mechanism for the development of prostate cancer involves modulation in the function of androgen receptor and its signaling pathways, we further studied the effect of WIN-55,212-2 on androgen receptor protein and mRNA expression and its subsequent effect on PSA production. Our results indicate that WIN-55,212-2 treatment significantly decreases androgen receptor protein (Fig. 3*A*) and mRNA expression (Fig. 3*C*) in LNCaP cells.

PSA belongs to the kallikrein family (17), is a serine protease with highly prostate-specific expression, and is the most widely employed marker in the detection of early prostate cancer. For these reasons, it is considered that agents which could reduce PSA levels may have important clinical implications for prostate cancer. Earlier studies reported that PSA is primarily regulated by androgens (24). This observation was based on the fact that the antiandrogen, cyperoterone acetate, had the ability to induce PSA, and that hydroxyflutamide could block androgen and progesterone induction of PSA glycoprotein, thus suggesting that PSA glycoprotein expression is influenced predominantly by androgens via its receptor, and the mutation of the receptor can affect the expression of this gene by steroids other than androgens (24). Recent studies have established that androgen receptor functions as a transcriptional regulator via its binding to androgen response elements within promoter and enhancer regions of PSA. PSA is currently the most accepted marker for assessment of prostate cancer progression in humans and is being detected in the serum of patients with prostate diseases including prostatitis, benign prostatic hypertrophy, and prostate cancer (18). It is reported that in LNCaP cells, androgens regulate PSA glycoprotein expression and mRNA via androgen receptor (25, 26). Our studies show a significant decrease

in intracellular, mRNA (Fig. 3*D*), as well as secreted levels of PSA by WIN-55,212-2 treatment of cells, suggesting that cannabinoid receptor agonists may be exploited to prevent prostate cancer progression.

PCNA recognizes nuclear antigens and its overexpression is associated with increase in PSA serum levels (27). PCNA expression has significant prognostic value and it seems to be a significant biomarker in prognosis and treatment of prostate cancer (27). Our results also suggest that concomitant with the decrease in PCNA protein expression (Fig. 4*A*), there was a decrease in PSA serum levels following WIN-55,212-2 treatment (Fig. 3*E*).

VEGF is a ubiquitous cytokine that regulates embryonic vasculogenesis and angiogenesis. Normal prostate epithelium expresses low levels of VEGF, whereas premalignant lesions have increased VEGF expression, which is additionally increased in prostate carcinoma (28). Studies have shown that cannabinoid treatment markedly reduced the expression of VEGF in gliomas, the most potent proangiogenic factor and also of angiopoietin 2, which contributes to the angiogenic process by preventing vessel maturation (29). Our results showed that treatment of LNCaP with WIN-55,212-2 inhibits growth and VEGF protein expression (Fig. 4*A* and *B*).

Recently, cannabinoids have received considerable attention due to their diverse pharmacologic activities such as cell growth inhibition, anti-inflammatory effects, and tumor regression. Our results suggest that treatment of androgen-responsive human prostate carcinoma LNCaP cells resulted in a decrease in intracellular and secreted levels of PSA, with concomitant inhibition of androgen receptor, cell growth, and induction of apoptosis. We conclude that cannabinoids should be considered as agents for the management of prostate cancer. If our hypothesis is supported by *in vivo* experiments, then the long-term implications of our work could be to develop non–habit-forming cannabinoid agonist(s) for the management of prostate cancer.

Acknowledgments

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Appendix 2

Cannabinoid Receptor Agonist-induced Apoptosis of Human Prostate Cancer Cells LNCaP Proceeds through Sustained Activation of ERK1/2 Leading to G_1 Cell Cycle Arrest*

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We have recently shown that the expression levels of both cannabinoid receptors CB_1 and CB_2 are higher in human prostate cancer cells than in normal prostate epithelial cells, and treatment of LNCaP cells with WIN-55,212-2 (a mixed CB_1/CB_2 agonist) resulted in inhibition of cell growth and induction of apoptosis (Sarfaraz, S., Afaq, F., Adhami, V. M., and Mukhtar, H. (2005) *Cancer Res.* 65, 1635–1641). This study was conducted to understand the mechanistic basis of these effects. Treatment of LNCaP cells with WIN-55,212-2 (1–10 μM; 24 h) resulted in: (i) an arrest of the cells in the G_0/G_1 phase of the cell cycle; (ii) an induction of p53 and p27/KIP1; (iii) down-regulation of cyclins D1, D2, E; (iii) decrease in the expression of cdk-2, -4, and -6; (iv) decrease in protein expression of pRb; (v) down-regulation of E2F (1–4); and (vi) decrease in the protein expression of DP1 and DP2. Similar effects were also observed when androgen-independent PC3 cells were treated with WIN-55,212-2 (5–30 μM). We further observed sustained up-regulation of ERK1/2 and inhibition of PI3k/Akt pathways in WIN-55,212-2-treated cells. Inhibition of ERK1/2 abrogated WIN-55,212-2-induced cell death suggesting that sustained activation of ERK1/2 leads to cell cycle dysregulation and arrest of cells in G_0/G_1 phase subsequently leading to an induction of apoptosis. Further, WIN-55,212-2 treatment of cells resulted in a dose-dependent increase in Bax/Bcl-2 ratio in such a way that favors apoptosis. The induction of apoptosis proceeded through down-regulation of caspases 3, 6, 7, and 9 and cleavage of poly (ADP-ribose) polymerases. Based on these data we suggest that cannabinoid receptor agonists should be considered as novel agents for the management of prostate cancer.

Prostate cancer (CaP)[2] ranks as the most common noncutaneous malignancy and the second leading cause of cancer-related deaths in American males, with similar trends in many Western countries. According to an estimate of the American Cancer Society, a total of 234,460 men will be diagnosed with CaP in the United States in the year 2006 and 27,350 CaP-related deaths are predicted (1). The major cause of mortality from this disease is metastasis of hormone refractory cancer cells that fail to respond to hormone ablation therapy (2, 3). Because surgery and current treatment options have proven to be inadequate in treating and controlling CaP, the search for novel targets and mechanism-based agents for prevention and treatment of this disease has become a priority.

In recent years, cannabinoids the active components of *Cannabis sativa linnaeus* (marijuana) and their derivatives are drawing renewed attention because of their diverse pharmacological activities such as cell growth inhibition, anti-inflammatory effects, and tumor regression (4–9). Further interest in cannabinoid research came from the discovery of the cannabinoid system and the cloning of specific cannabinoid receptors (10). Two cannabinoid receptors have been identified: the "central" CB_1 and the "peripheral" CB_2 receptor. In a recent study, we have shown that WIN 55,212-2 (Fig. 1) a mixed CB_1/CB_2 receptor agonist imparts cell growth inhibitory effects in LNCaP cells via an induction of apoptosis. An important observation of this study was that WIN 55,212-2 treatment did not result in apoptosis of the normal prostate epithelial cell at similar doses (11).

Here, we show that treatment of human prostate cancer LNCaP cells with cannabinoid receptor agonist WIN-55,212-2 resulted in an arrest of the cells in the G_0/G_1 phase of the cell cycle, and this arrest was associated with a sustained activation of ERK1/2, induction of p27/KIP1, and inhibition of cyclin D1. Blocking of both cannabinoid receptors CB_1 and CB_2 by their specific antagonist resulted in inhibition of ERK1/2 activation. Inhibition of ERK1/2 signaling by the ERK1/2 inhibitor PD98059 and its specific siRNA abrogated these effects.

EXPERIMENTAL PROCEDURES

Materials—R-(+)-WIN 55,212-2 (2,3 dihydro-5-methyl-3-([morpholinyl]methyl) pyrollo (1,2,3 de)-1,4-benzoxazinyl]-[1-napthaleny]methanone, $C_{27}H_{26}N_2O_3 \cdot CH_3SO_3H$ was purchased from Sigma. CB_1 receptor antagonist SR141716 (SR1) and CB_2 receptor antagonist SR144528 (SR2) were procured from Dr. Herbert H. Seltzman (NIDA, National Institutes of Health, Division of Neuroscience and Behavioral Research, through RTI International, Research Triangle Park, NC). ERK1/2 inhibitor PD98059 was purchased from Tocris Biosciences (Ellisville, MO). Dulbecco's modified Eagle's medium and fetal bovine serum were procured from Invitrogen. Antibi-

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[2] The abbreviations used are: CaP, prostate cancer; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; Rb, retinoblastoma.
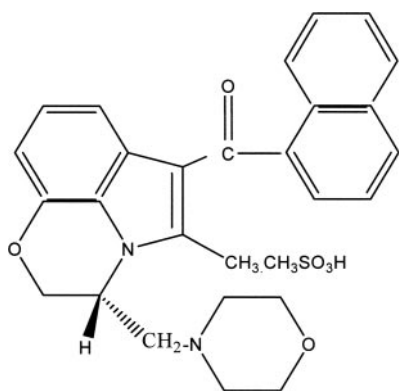
FIGURE 1. **Chemical structure of WIN-55,212-2 (2,3 dihydro-5-methyl-3 ([morpholinyl]methyl) pyrollo (1,2,3 de)-1,4-benzoxazinyl]-[1-naptha-leny] methanone.**

otics (penicillin and streptomycin) used were obtained from Cellgro Mediatech, Inc. (Herndon, VA). The mono- and poly-clonal antibodies (p53, cdk2, -4, and -6, KIP1/p27, E2F-3, and DP-2) were obtained from Santa Cruz Biotechnology Inc. The human reactive monoclonal and polyclonal antibodies (cyclins D1, D2, E, pRb, E2F-1, E2F-2, E2F-4, and DP-1) were obtained from Labvision (Fremont, CA). Monoclonal and polyclonal antibodies for anti-PARP, Bcl-2 Bax were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-PARP (116 kDa) and anti-PI3K (p85) was purchased from Upstate Biotechnology and anti-PARP (p85) was purchased from Promega (Madison, WI). Anti-phospho-ERK1/2 p42/44 was purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit secondary horseradish peroxidase conjugate was obtained from Amersham Biosciences. Protein was estimated using the BCA protein assay kit obtained from Pierce (Rockford, IL).

Cell Culture—LNCaP and PC3 cells obtained from ATCC (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. PC3 cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment.

Treatment of Cells—WIN-55,212-2, (dissolved in Me$_2$SO) was used for the treatment of cells. The final concentration of Me$_2$SO used was 0.1% (v/v) for each treatment. For dose-dependent studies, LNCaP cells were treated with WIN-55,212-2 at 1.0, 2.5, 5.0, 7.5, 10.0 μM final concentrations for 24 h in complete cell medium. PC3 cells were treated with WIN-55,212-2 at 5.0, 10, 20, 25, and 30 μM final concentration for 24 h in complete cell medium. Control cells were treated with vehicle alone. To establish the role of CB$_1$ and CB$_2$ receptor in WIN-55,212-2 induced ERK1/2 activation cells were pre-treated with 3 μM SR141716 and SR144528 alone, and in the second set, cells were pretreated with both the antagonists (3 μM each) for 3 h followed by incubation with 7.5 μM WIN-55,212-2 for 24 h. To study the role of ERK1/2 in cannabinoid receptor induced cell growth inhibition, cells were pretreated with 30 μM ERK1/2 inhibitor PD98059 for 1 h followed by incubation with 7.5 μM WIN-55,212-2 for 24 h.

Cell Viability—The cells were grown at density of 1×10^6 cells in 100-mm culture dishes and treated with WIN-55,212-2 (1–10 μM) for 24 h. The cells were trypsinized and collected in the microfuge tube. The cells were pelleted by centrifugation, and the cell pellet was resuspended in phosphate-buffered saline (PBS) (300 μl). Trypan blue (0.4% in PBS; 10 μl) was added to a smaller aliquot (10 μl) of cell suspension, and the number of cells (viable-unstained and nonviable-blue) were counted using a hemocytometer.

Quantification of Apoptosis and Cell Cycle Analysis by Flow Cytometry—The cells were grown at a density of 1×10^6 cells in 100-mm culture dishes and were treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, 10.0 μM doses) for 24 h. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by use of an Apo-direct apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) as per the manufacturer's protocol. The labeled cells were analyzed using a FACScan benchtop cytometer (BD Biosciences, San Jose, CA) at the UWCCC Flow Cytometry Facility in the University of Wisconsin. Results were analyzed using Mod-Fit LT software (Verity Software House, Topsham, ME) for cell cycle and WinMD1 version 2.8 software for quantification of apoptosis.

Detection of Cleaved Caspase-3 by Confocal Microscopy—The cells were grown in two chambered cell culture slides (BD Biosciences), treated with WIN-55,212-2 (5.0, 7.5, 10.0 μM doses) for 24 h, washed with 1× PBS at room temperature, and were immediately fixed in cold 100% methanol at −20 °C for 10 min. Cells were blocked with blocking buffer (5.5% normal goat serum in TBST, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) for 60 min, and were washed with TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). Cells were then incubated with primary antibody Alexa Fluor 488 conjugate, (Cell Signaling Technology) overnight using the vendor's protocol. After incubation, cells were washed twice for 5 min with TBST and once with TBS. Coverslips were mounted using the Prolong Antifade kit obtained from Molecular Probes, (Eugene, OR). Cells were visualized with a Bio-Rad MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200 microscope at the Keck Neural Imaging Laboratory in the University of Wisconsin, Madison.

Silencing of ERK 44/42 by Small Interfering RNA—For suppressing ERK1/2 expressions, ERK1, ERK2, and control scrambled siRNA were purchased from Cell Signaling Technology (Danvers, MA). LNCaP cells were transfected with siRNAs (ERK1, 150 nmol/liter and ERK2, 80 nmol/liter, scrambled siRNA 150 nmol/liter) using the nucleofection kit R specific for LNCaP transfection from Amaxa Biosystems (Gaithersburg, MD). Cells were resuspended in a solution from nucleofector kit R following the manufacturer's guidelines. 100 μl of nucleofector solution R was mixed with 2×10^6 cells and siRNA. They were then transferred to the cuvette provided with the kit and was nucleofected with an Amaxa Nucleofector apparatus. Cells were transfected using the T-001 pulsing parameter and were transferred into 100-mm plates containing 37 °C prewarmed culture medium. After transfection, cells were cultured for 48 h, after which the medium was
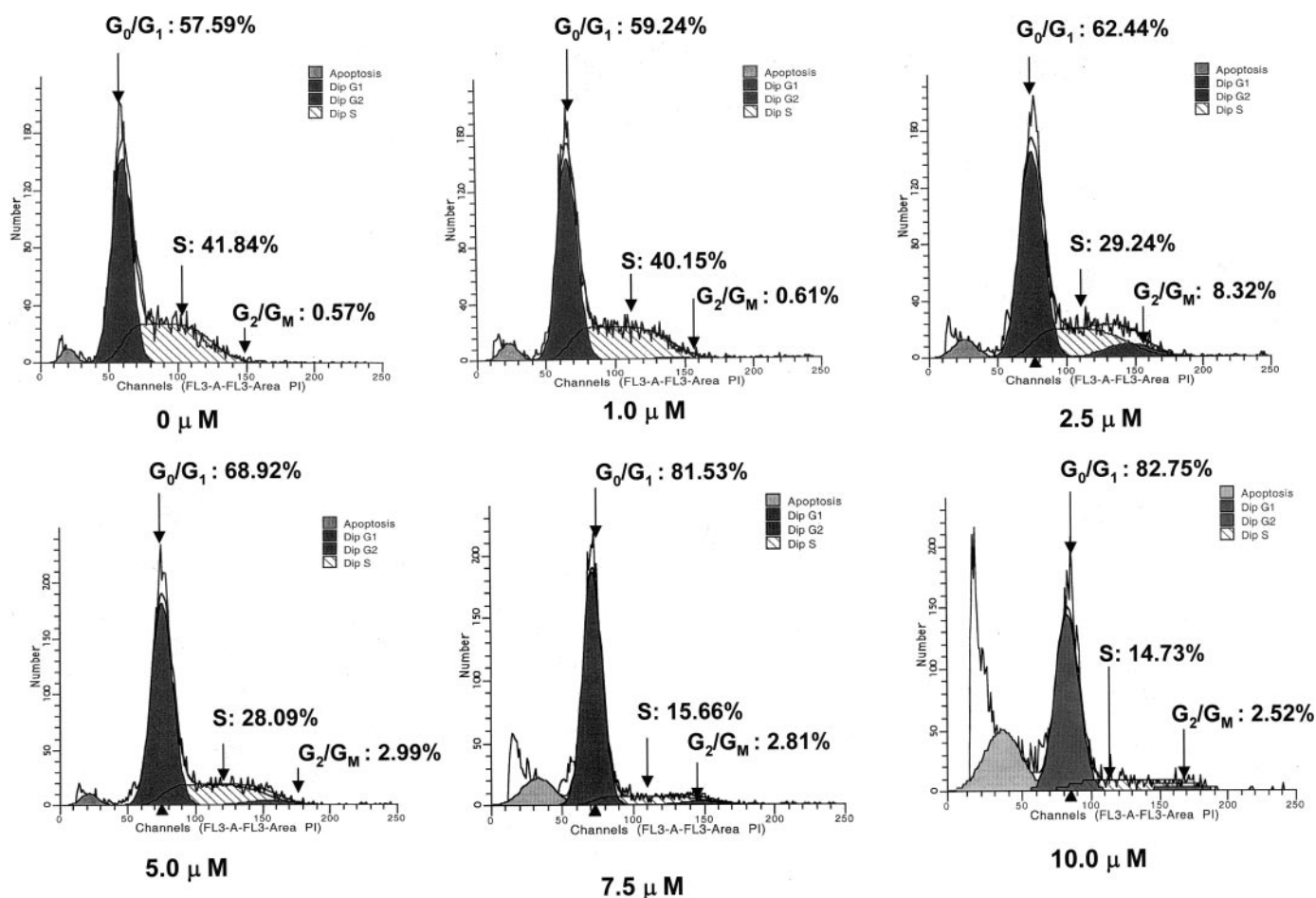
FIGURE 2. **Effect of WIN-55,212-2 treatment on cell cycle in LNCaP cells.** Cell cycle analysis was performed by flow cytometry as detailed under "Experimental Procedures." The labeled cells were analyzed using a FACScan benchtop cytometer, and the percentage of cells in the G_0–G_1, S, and G_2-M phases were calculated using ModFit LT software. The data shown here are from a typical experiment repeated three times.

replaced with fresh medium, cells were treated with 7.5 μM WIN-55,212-2 for 24 h, and protein lysates were prepared. Using 2 μg of GFP we observed 70–80% transfection efficiency with this protocol.

Preparation of Cell Lysates and Western Blot Analysis—Following treatment of cells with WIN-55,212-2, the medium was aspirated, and the cells were washed with cold PBS (10 mmol/liter, pH 7.45). The cells were then incubated in ice-cold lysis buffer (50 mmol/liter Tris-HCl, 150 mmol/liter NaCl, 1 mmol/liter EGTA, 1 mmol/liter EDTA, 20 mmol/liter NaF, 100 mmol/liter Na_3VO_4, 0.5% Nonidet P-40, 1% Triton X-100, 1 mmol/liter phenylmethylsulfonyl fluoride (pH 7.4), with freshly added protease inhibitor mixture (Protease Inhibitor Mixture Set III, Calbiochem, La Jolla, CA) over ice for 20 min. The cells were scraped, and the lysate was collected in a microcentrifuge tube and passed through a 21.5-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14,000 $\times$ g for 15 min at 4 °C, and the supernatant (total cell lysate) collected, aliquoted, and was used on the day of preparation or immediately stored at −80 °C for use at a later time. For Western blotting, 25–50 μg protein was resolved over 12% polyacrylamide gels and transferred onto a nitrocellulose membrane. The nonspecific sites on blots were blocked by incubating in blocking

buffer (5% nonfat dry milk/1% Tween 20 in 20 mmol/liter TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate monoclonal primary antibody in blocking buffer for 90 min to overnight at 4 °C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate and detected by chemiluminescence and autoradiography using hyperfilm obtained from Amersham Biosciences (UK Ltd.). Densitometric measurements of the bands in Western blot analysis were performed using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT).

Statistical Analysis—Results were analyzed using a two-tailed Student's t test to assess statistical significance. Values of $p < 0.05$ were considered statistically significant.

RESULTS

WIN-55,212-2 Causes G_1 Phase Cell Cycle Arrest—We have earlier shown that treatment of LNCaP cells with WIN-55,212-2 (1–10 μM) for 24 h significantly decreased the cell viability and led to induction of apoptosis (11). Several studies have shown that the induction of apoptosis may be cell cycle-dependent (12–16). Therefore, in the next series of experiments, we tested the hypothesis that WIN-55,212-2-caused apoptosis of LNCaP cells is mediated via cell cycle
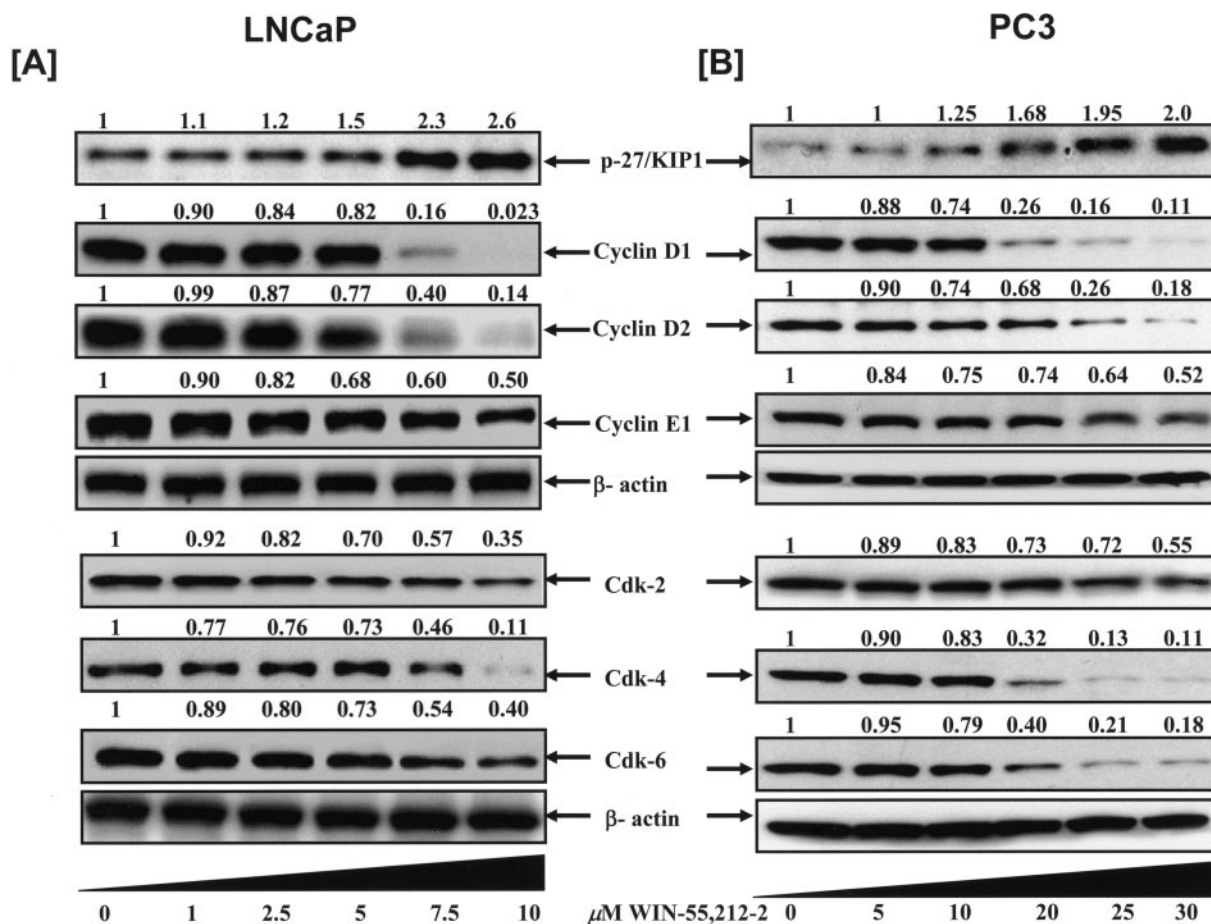
FIGURE 3. *A*, effect of WIN-55,212-2 treatment on the protein expression of KIP1/p27, cyclin D1, D2, and E and cdk 2, 4, and 6 in LNCaP cells. *B*, effect of WIN-55,212-2 treatment on the protein expression of KIP1/p27, cyclin D1, D2, and E and cdk 2, 4, and 6 in PC3 cells. As detailed under "Experimental Procedures," the cells were treated with Me$_2$SO alone or specified concentrations of WIN-55,212-2, and total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

blockade. We performed DNA cell cycle analysis to assess the effect of WIN-55,212-2 treatment on the distribution of cells in the cell cycle. As shown in Fig. 2, compared with vehicle treatment, WIN-55,212-2 treatment resulted in a dose-dependent accumulation of cells in G_1 phase of the cell cycle (59, 62, 69, 81, and 83% cells in G_1 phase at 1.0, 2.5, 5.0, 7.5, and 10 μM concentrations, respectively). This observation is important because the molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (17, 18). Consistent with this observation, in recent years, inhibition of the cell cycle has been appreciated as a target for the management of cancer (19, 20).

WIN-55,212-2-induced Cell Cycle Arrest Is Mediated via an Induction of KIP1/p27 and Concomitant Inhibition in Cyclins D1, D2, E, and Cdk2, Cdk4, and Cdk6—Because our studies demonstrated that WIN-55,212-2 treatment of cells resulted in a G_1-phase cell cycle arrest and apoptosis, we examined the effect of WIN-55,212-2 on cell cycle regulatory molecules operative in G_1 phase of the cell cycle. Studies have shown a critical role of p27/KIP1 in apoptosis and cell cycle progression through G_0–G_1 phase (21–23). We observed a significant induction of p27/KIP1 by WIN-

55,212-2 at 5–10 μM doses. (Fig. 3*A*). Relative density data revealed 1.5-, 2.3-, and 2.6-fold increases in the protein expression of Kip/p27 at 5.0, 7.5, and 10 μM concentrations of WIN-55,212-2, respectively. Using immunoblot analysis, we also assessed the effect of WIN-55,212-2 treatment on the protein expression of the cyclins and cdks, which are known to be regulated by KIP1/p27. WIN-55,212-2 treatment of the cells resulted in a dose-dependent decrease in protein expression of cyclin D1, cyclin D2, and cyclin E (Fig. 3*A*) as well as cdk2, cdk4, and cdk6 (Fig. 3*A*). Densitometric analysis data of cyclins revealed a significant decrease in the expression of cyclin D1 (84%, 97%), cyclin D2 (60%, 86%), and cyclin E (40%, 50%) at 7.5 and 10.0 μM concentrations of WIN-55,212-2, respectively (Fig. 3*B*). Relative density data of cdks also revealed a significant decrease in the expression of cdk2 (43%, 65%), cdk4 (54%, 89%), and cdk6 (46%, 60%) at similar doses of WIN-55,212-2. In the next series of experiments we assessed the effect of WIN-55,212-2 on p27/KIP1, cyclin and cdk in androgen-insensitive cell PC3. Cells were treated with different doses of WIN-55,212-2 (5, 10, 20, 25, 30 μM), and we found an induction in p27/KIP1 and down-regulation in the protein expression of cyclin and cdk particularly at doses of 20–30 μM (Fig. 3*B*).
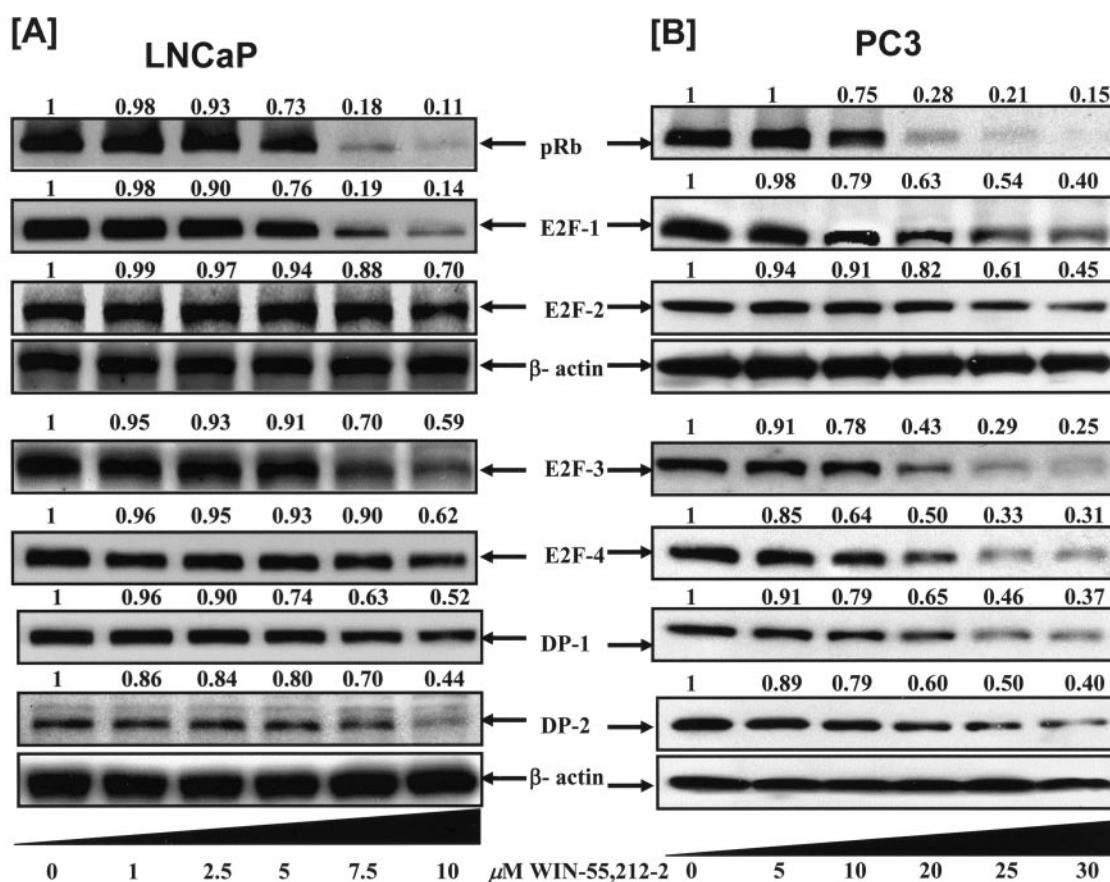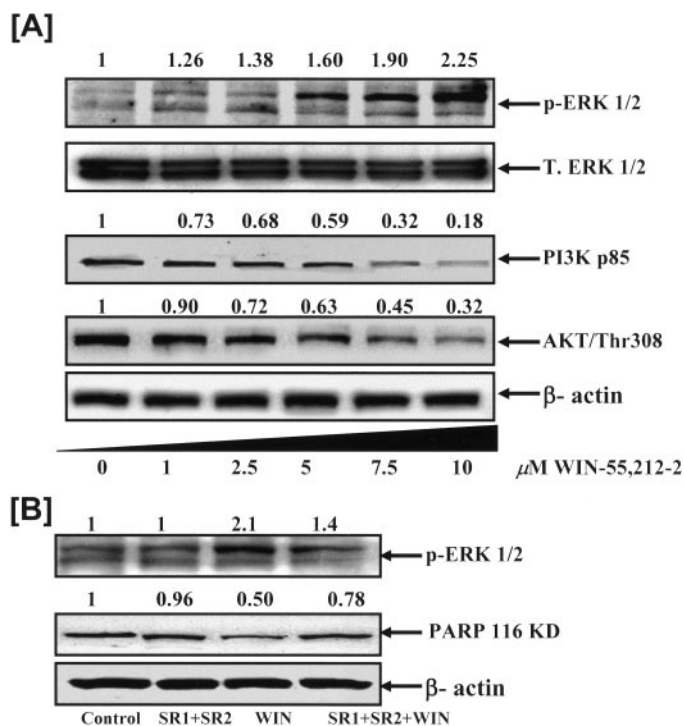
FIGURE 4. *A*, effect of WIN-55,212-2 treatment on protein expression of pRb, E2F (1–4), DP1, and DP2 in LNCaP cells. *B*, effect of WIN-55,212-2 treatment on protein expression of pRb, E2F (1–4), DP1 and DP2 in PC3 cells. As detailed under "Experimental Procedures," the cells were treated with Me₂SO alone or specified concentrations of WIN-55,212-2, and total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

WIN-55,212-2 Inhibits Protein Expression of pRB, E2F, and DP—Down-regulation of cdk4/6 has been shown to be associated with a decrease in the expression of retinoblastoma (pRb) tumor suppressor protein a key regulator of the $G_1 \rightarrow$ S phase transition in the cell cycle (24, 25). Therefore, we next examined the effect of WIN-55,212-2 on protein expression of pRb. Immunoblot data revealed that WIN-55,212-2 treatment of cells resulted in a significant decrease in the protein expression of pRb. Densitometric analysis of immunoblots showed 27, 82, and 89% inhibition at 5.0, 7.5, and 10 μM concentrations of WIN-55,212-2 (Fig. 4*A*). Because pRb controls cell cycle by binding to and inhibiting the E2F transcription factors, we determined the protein expression of E2F (1–4) transcription factors. As shown in Fig. 4*A*, WIN-55,212-2 treatment of cells resulted in a dose-dependent decrease in E2F transcription factors. Relative density data revealed an inhibition in E2F-1 (81 and 86%), E2F-2 (12 and 30%), E2F-3 (30 and 41%), and E2F-4 (10 and 38%) at a concentration of 7.5 and 10 μM WIN-55,212-2. Because the activity of E2F is known to be dependent on its heterodimeric association with members of DP family of proteins, we also evaluated the effect of WIN-55,212-2 treatment on both members of DP family viz. DP-1 and DP-2. Immunoblot and densitometric analysis data revealed a decrease in the protein expression of DP-1 (37 and 48%) and DP-2 (30 and 56%) at 7.5 and 10 μM concentration of WIN-55,212-2 (Fig. 4*A*). In the next series of experiments we assessed

the effect of WIN-55,212-2 on pRB, E2F family of proteins (1–4) and its heterodimeric partners DP-1 and DP-2 in androgen-insensitive cell PC3. Cells were treated with different doses of WIN-55,212-2 (5, 10, 20, 25, 30 μM), and we found a decrease in the protein expression of pRB, E2F (1–4), DP-1, and DP-2 at 20–30 μM doses (Fig. 3*B*).

WIN-55,212-2-induced Sustained Activation of ERK and Inhibition of PI3K/AKT Leads to Apoptosis through Cannabinoid Receptors—It has been reported that challenging gliomas with cannabinoids leads to the activation of ERK1/2 signaling and AKT inhibition (26, 4). This sustained ERK1/2 activation can mediate cell cycle arrest (8). We observed a significant and sustained activation of ERK1/2 and significant inhibition of PI3K (p85) and AKT (Thr[308]) when LNCaP cell were treated with WIN-55,212-2 at a dose of 1–10 μM (Fig. 5*A*). To confirm that ERK1/2 activation is cannabinoid receptor-mediated, cells were pretreated with 3 μM SR141716 (CB₁ antagonist) and SR144528 (CB₂ antagonist) for 3 h followed by treatment with WIN-55,212-2. Data in Fig. 5*B* show that there was no activation of ERK1/2 when treated with the antagonists alone. WIN-55,212-2 (7.5 μM) treatment resulted in significant activation of ERK1/2. When antagonist were coadministered with WIN-55,212-2, there was a decrease in the protein expression of ERK1/2 and a significant increase in the protein expression of PARP (116) as compared with the treatment 7.5 μM WIN-55,212-2 alone (Fig. 5*B*). These data suggest that sustained

[A]

[B]

FIGURE 5. **Effect of WIN-55,212-2 treatment on protein expression.** *A*, ERK1/2 (phospho-p44/42, Thr^{202}/Tyr^{204}), anti-PI3K kinase (p85), and AKT (Thr^{308}) in LNCaP cells. *B*, effect of CB$_1$ (SR141716) and CB$_2$ (SR144528) antagonist on protein expression of ERK1/2 and PARP cleavage (116 kDa) in LNCaP cells. As detailed under "Experimental Procedures," the cells were treated with 7.5 μM concentrations of WIN-55,212-2 and 3 μM CB$_1$ (SR141716) and CB$_2$ (SR144528). Total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin using UN-SCAN-IT software (Silk Scientific, Inc.). The data shown here are from a representative experiment repeated three times with similar results.

ERK1/2 activation and subsequent apoptosis is mediated through cannabinoid receptors.

WIN-55,212-2-induced Sustained Activation of ERK1/2 Leads to Cell Growth Inhibition with the Induction of Apoptosis and Cell Cycle Arrest—To define the role of ERK1/2 in cannabinoid receptor-induced cell growth inhibition and apoptosis, LNCaP cells were pretreated with ERK1/2 inhibitor PD98059 (30 μM) for 1 h. This treatment alone resulted in no change in the morphology of the cells. However, 7.5 μM WIN-55,212-2 treatment resulted in distinct morphological changes in LNCaP cells, as cells became round and detached from the surface of the plate, whereas pretreatment of LNCaP cells with PD98059 (30 μM) prevented these morphological changes (Fig. 6*A*). WIN-55,212-2 treatment of LNCap cells results in G$_1$ cell cycle arrest. To assess whether cell cycle arrest is mediated via activation of ERK1/2, we next performed DNA cell cycle analysis. As shown in Fig. 6*B*, blocking of ERK1/2 activation by its inhibitor PD98059 resulted in a decrease in the number of cells in the G$_1$ phase of cell cycle (72%) when compared with WIN-55,212-2 treatment alone (81%). To assess whether cell cycle dysregulation leads to induction of apoptosis, we next quantified the extent of apoptosis by flow cytometric analysis. As shown in Fig. 6*C*, WIN-55,212-2 treatment of LNCaP cells at a dose of 7.5 μM resulted in 23% of apoptotic cells. Apoptosis was only 9% when WIN-55,212-2 (7.5 μM) was coadministered with

PD98059 (30 μM). We next determined whether PD98059 reversed the activation of ERK1/2 by WIN-55,212-2 treatment alone, and we found that ERK1/2 protein expression was significantly decreased when WIN-55,212-2 was given in combination with PD98059 (Fig. 6*D*). We next determined the effect of PD98059 on p27/KIP1 (Fig. 6*D*), a cell cycle regulatory molecule operative in G$_1$ phase of the cell cycle, and cyclin D1 because of its function in influencing cell proliferation. WIN-55,212-2 treatment increased the protein expression of p27/KIP1 whereas this increase in expression was down-regulated when WIN-55,212-2 was given in combination with PD98059. WIN-55,212-2 treatment significantly inhibited the expression of cyclin D1, and this effect was significantly reversed (>55%) when WIN-55,212-2 was coadministered with PD98059 (Fig. 6*D*). We also observed a decrease (62%) in the protein expression of BCl-2, a pro-apoptotic protein when the cells were treated with WIN-55,212-2 at 7.5 μM; this effect was significantly reversed to 50% when WIN-55,212-2 was coadministered with ERK1/2 inhibitor (Fig. 6*D*).

To further validate the role of ERK1/2 in WIN-55,212-2-induced cell cycle arrest leading to apoptosis, we silenced ERK1/2 by using small interfering RNA against ERK1/2. We observed that WIN-55,212-2 did not induce ERK1/2 activation and p27 when ERK1/2 was silenced (Fig. 6*E*). Similarly, protein expression of cyclin D1 and Bcl-2 which down-regulated by WIN-55,212-2 was found to be reversed when ERK1/2 was silenced.

WIN-55,212-2 Induces Apoptosis via the Classical Apoptotic Pathway—The above data suggest that WIN-55,212-2 induces growth inhibition via cell cycle arrest in G$_1$ phase of the cell cycle followed by apoptosis. Because p53 is one of the major regulators of apoptosis, expression of this tumor suppressor sensitizes cells to apoptosis in response to stress. We observed a significant up-regulation in the protein expression of p53 when cells were treated with WIN-55,212-2 (Fig. 7*A*). p53-induced apoptosis results from overlapping downstream pathways that suppress mitogenic and survival signaling and promote pro-apoptotic signaling. In this context, p53 can up-regulate the pro-apoptotic Bcl-2 family member Bax and possibly transcriptionally repress the anti-apoptotic protein Bcl-2. Because Bax and Bcl-2 plays a crucial role in apoptosis, we next determined the effect of WIN-55,212-2 treatment of LNCaP cells on protein levels of Bax and Bcl-2. The Western blot analysis exhibited a significant increase in the protein expression of Bax at 7.5 and 10 μM concentrations of WIN-55,212-2 (Fig. 7*A*). In sharp contrast, the protein expression of Bcl-2 was significantly decreased by WIN-55,212-2 treatment in a dose-dependent fashion (Fig. 7*A*). A significant dose-dependent shift in the ratio of Bax to Bcl-2 was observed after WIN-55,212-2 treatment indicating the induction of apoptotic process (Fig. 7*B*). Relative density data revealed an increase in protein expression of Bax by 2.1- and 2.9-fold with concomitant decrease in Bcl-2 protein expression by 71 and 79% at a dose of 7.5 and 10 μM, respectively. A decrease in Bcl-2 expression was associated with an increase in AIF to 2.0- and 2.1-fold at the above mentioned doses of WIN-55,212-2 (Fig. 7*A*).

Alteration in Bax/Bcl-2 is known to initiate caspase signaling; therefore, we evaluated the involvement of various caspases

during WIN-55,212-2-mediated apoptotic death of LNCaP cells. As shown by the immunoblot analysis, WIN-55,212-2 treatment was found to result in a significant decrease in the pro form of caspase-3 (Fig. 7*C*), caspase-6, caspase-7, and caspase-9 (Fig. 7*D*) at a concentration of 7.5 and 10 μM. To assess possible involvement of caspase-3 activation in apoptosis, we next measured cleaved caspase-3 by immunoblot analysis and immunostaining (Fig. 7*C*). Cells were stained with Alexa Fluor 488 conjugate antibody and were viewed under confocal microscope. Intensity of the active caspase-3 staining was higher in cells treated with 7.5 and 10 μM concentrations of WIN-55,212-2 compared with that at lower concentrations of WIN-55,212-2 and control (Fig. 7*C*). The downstream signals during apoptosis are transmitted via caspases, which upon conversion from pro to active forms mediate the cleavage of PARP.

We found that WIN-55,212-2 treatment caused cleavage of 116 kDa PARP to 85 kDa (Fig. 7*E*). Relative density data revealed a decrease in the protein expression of PARP (116 kDa) (49 and 81%) with a concomitant increase in its cleaved product (85 kDa) by 3.1- and 4.4-fold at concentrations of 7.5 and 10 μM, respectively.

DISCUSSION

Cannabinoids and their derivatives are drawing considerable attention in the treatment of cancer because of their diverse activities such as cell growth inhibition, anti-inflammatory effects, and tumor regression (6, 7). Accumulated evidence indicates that cannabinoid receptor(s) could be an important target for the treatment of cancer (27, 28, 29). We have earlier shown that WIN-55,212-2 induced apoptosis of
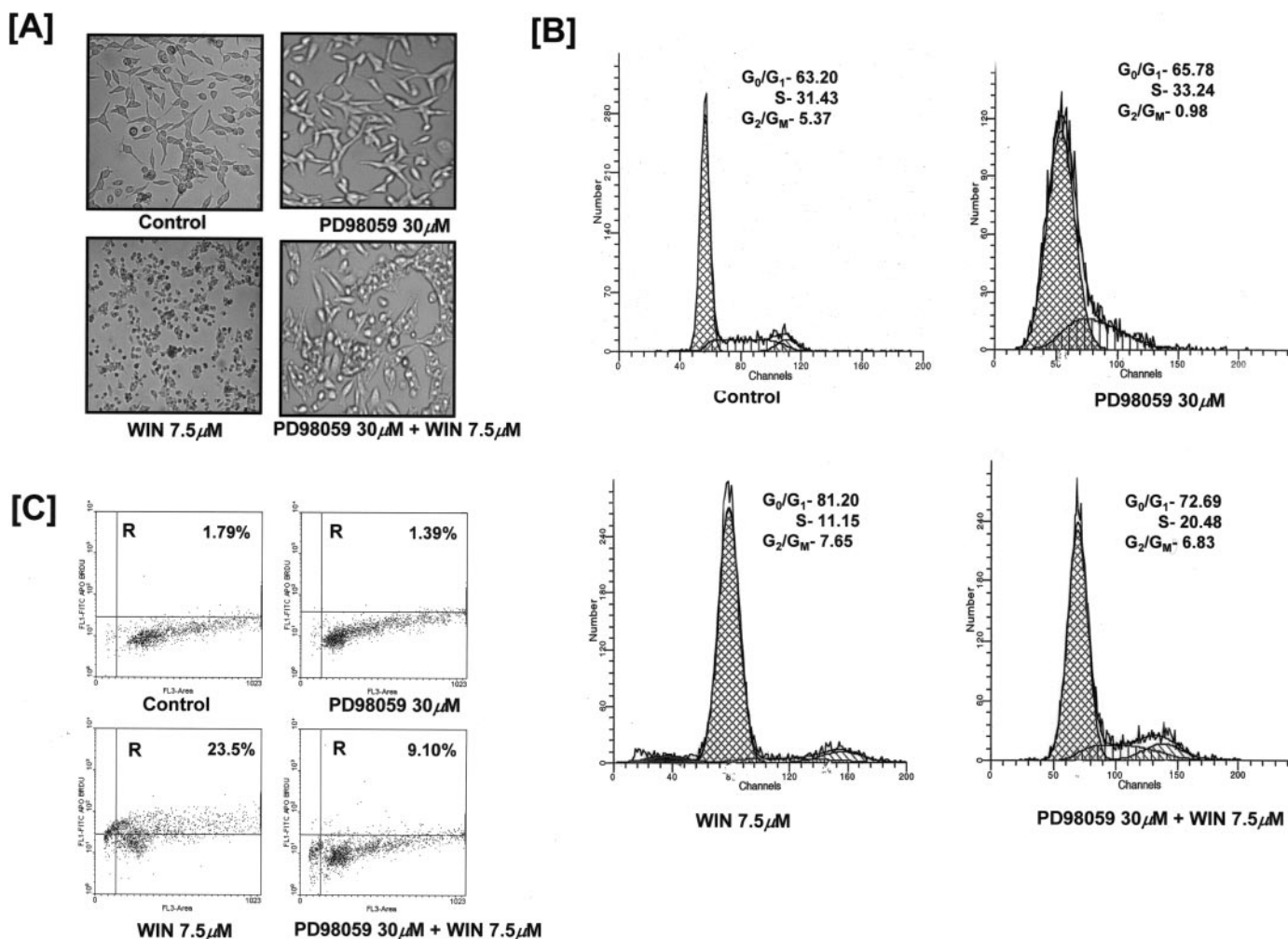
FIGURE 6. **Effect of simultaneous treatment of WIN-55,212-2 and ERK1/2 inhibitor.** *A*, morphology of LNCaP cells; *B*, cell cycle in LNCaP cells. Cell cycle analysis was performed by flow cytometry as detailed under "Experimental Procedures." The labeled cells were analyzed using a FACScan benchtop cytometer and the percentage of cells in G$_0$–G$_1$, S, and G$_2$-M phases were calculated using ModFit LT software. The data shown here are from a typical experiment repeated three times. *C*, quantification of apoptosis by flow cytometry. Cells showing fluorescence (*R*) are considered as apoptotic, and their percentage population is indicated. Data from representative experiments repeated thrice with similar results. *D*, protein expression of p27/KIP1, cyclin D1, and Bcl-2 in LNCaP cells. As detailed under "Experimental Procedures," the cells were treated with 7.5 μM concentrations of WIN-55,212-2 and 30 μM ERK1/2 inhibitor PD98059. Total cell lysates were prepared for immunoblot analysis. The bar diagram represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results. *, $p < 0.01$ compared with WIN; **, $p < 0.001$ compared with control. *E*, silencing of ERK1/2 prevents activation of ERK1/2 and p27 and reverses down-regulation of cyclin D1 and Bcl-2. LNCaP cells transfected with 150 nM ERK1, 80 nM ERK2 or scrambled siRNA (150 nM) for 48 h and were then treated with 7.5 μM WIN-55,212-2 for 24 h. Cell lysates were analyzed by immunoblotting using antibodies against ERK1/2 (phospho-p44/42, Thr^{202}/Tyr^{204}), p27, cyclin D1, Bcl-2, and β-actin. The bar diagram represents relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results. *, $p < 0.01$; #, $p < 0.001$ compared with WIN; **, $p < 0.001$ compared with control.
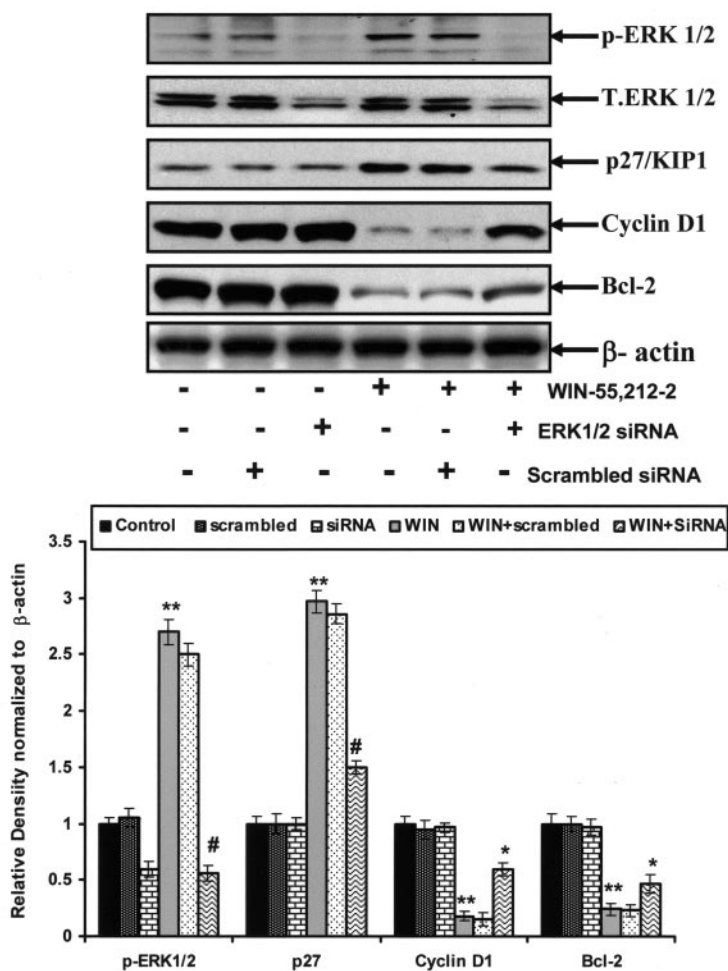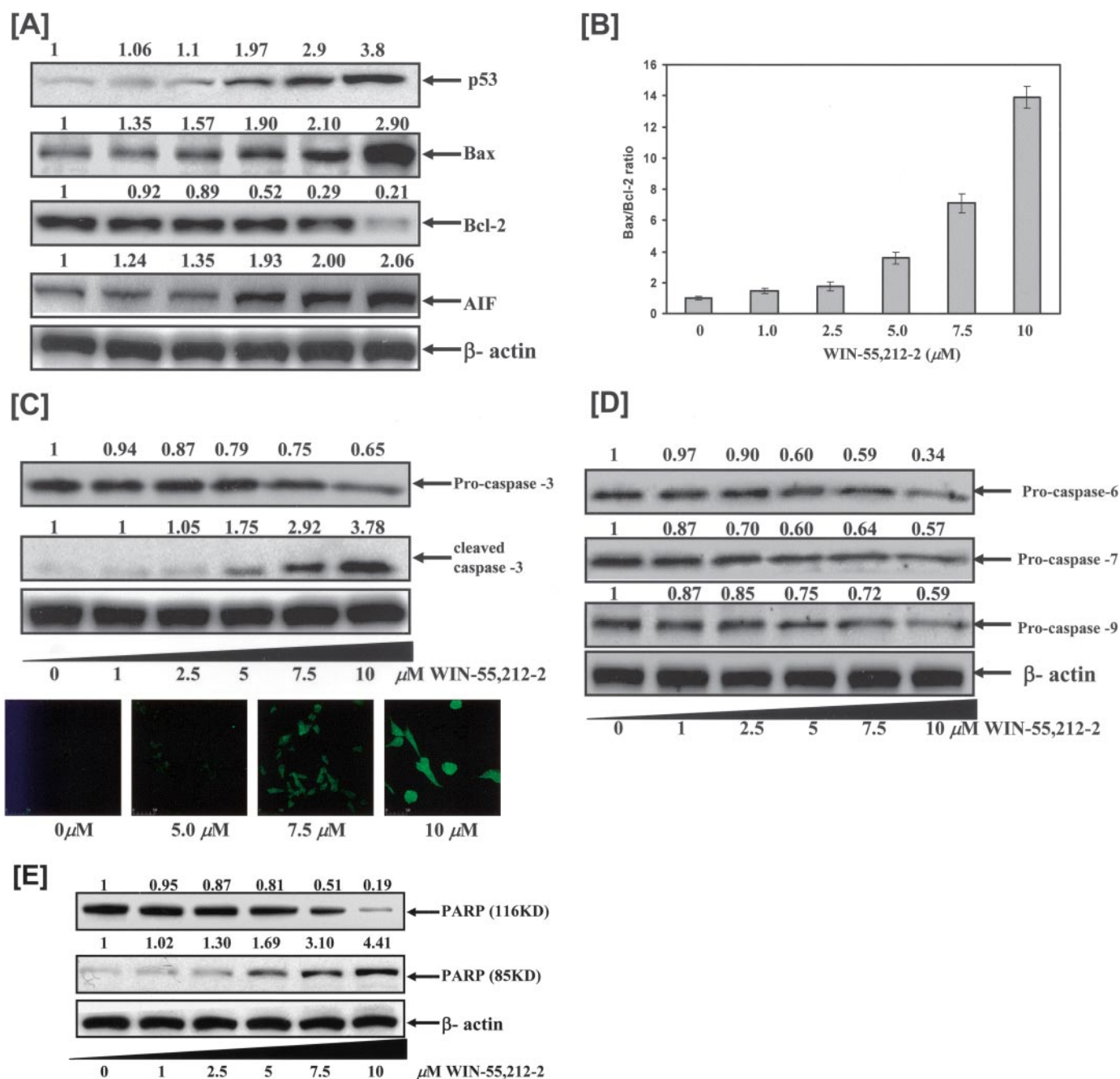
[D]
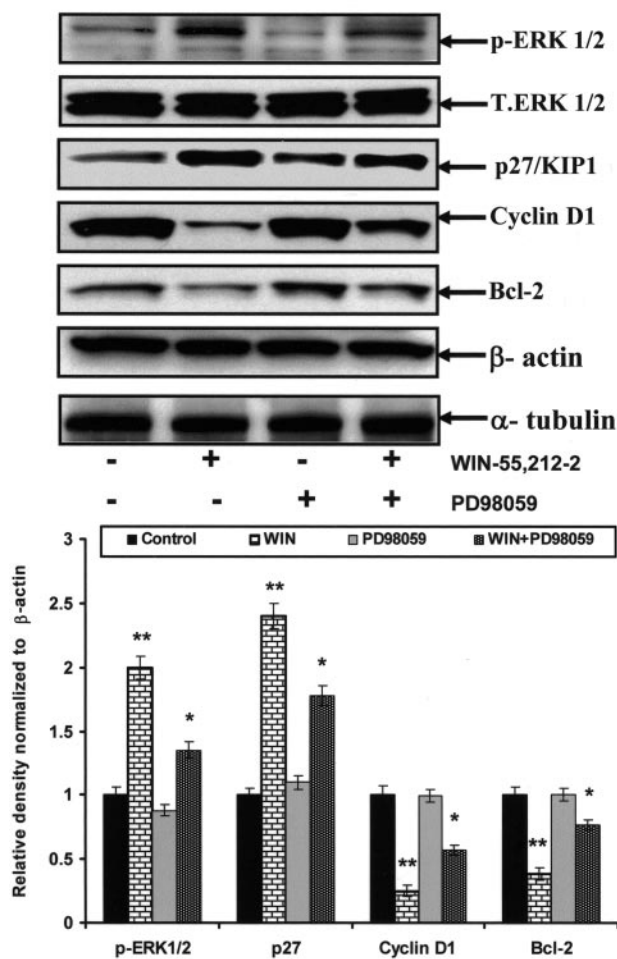
[E]

FIGURE 6—*continued*

prostate cancer LNCaP cells is mediated through CB_1 and CB_2 receptors and suggested that these receptors could be an important targets for the treatment of prostate cancer (11). The present study was designed to define the mechanism(s) of the antiproliferative and proapoptotic effects of cannabinoid receptor agonist WIN-55,212-2 against prostate cancer. We provide evidence that WIN-55,212-2 treatment of LNCaP cells activates ERK1/2 leading to cell cycle dysregulation and G_1 arrest, which in turn up-regulates the Bax/BCl-2 ratio and activates caspases resulting in an induction of apoptosis (Fig. 8).

It is well established that uncontrolled cellular growth as a consequence of defects in cell cycle and apoptotic machinery, is responsible for the development of most of the cancers including prostate cancer. Therefore those agents that can modulate apoptosis in cancer cells may be able to affect the steady state cell population and be useful in the management and therapy of cancer. This notion assumes importance because in cancer a time balance between proliferation and apoptosis is lost which has been implicated in cellular mass and tumor progression. Consistent with this notion, there is a need to develop novel targets and mechanism-based apoptosis inducing agents for the

management of prostate cancer. One of the most exciting and promising areas of current cannabinoid research is the ability of these compounds to control the cell survival/death decision (8). Several studies have shown that the induction of apoptosis may be cell cycle dependent (12–16). Therefore, we determined whether WIN-55,212-2-induced apoptosis of LNCaP cells is mediated via cell cycle blockade. We therefore analyzed the effect of WIN-55,212-2 treatment on the distribution of cells in different phases of the cell cycle. As shown in Fig. 2, WIN-55,212-2 treatment was found to result in dose-dependent accumulation of cells in G_1 phase of the cell cycle. In recent years, inhibition of the cell cycle has been appreciated as target for the management of cancer (19, 20). We next studied the involvement of CKI-cyclin-CDK machinery operative in G_1-phase of cell cycle arrest in LNCaP cells by WIN-55,212-2 treatment. The cell cycle in eukaryotes is regulated by members of protein kinase complexes. Each complex is composed minimally of cyclins (regulatory subunit) that bind to cdks (catalytic subunit) to form active cyclin-cdk complexes. These complexes are activated at different checkpoints after certain intervals during the cell cycle and can also be regulated by several exogenous factors (17). Cdk activity is additionally regulated by

[A]

| 1 | 1.06 | 1.1 | 1.97 | 2.9 | 3.8 |

→ p53

| 1 | 1.35 | 1.57 | 1.90 | 2.10 | 2.90 |

→ Bax

| 1 | 0.92 | 0.89 | 0.52 | 0.29 | 0.21 |

→ Bcl-2

| 1 | 1.24 | 1.35 | 1.93 | 2.00 | 2.06 |

→ AIF

→ β-actin

[B]

[C]

| 1 | 0.94 | 0.87 | 0.79 | 0.75 | 0.65 |

→ Pro-caspase -3

| 1 | 1 | 1.05 | 1.75 | 2.92 | 3.78 |

→ cleaved caspase -3

0   1   2.5   5   7.5   10   μM WIN-55,212-2

0μM   5.0 μM   7.5 μM   10 μM

[D]

| 1 | 0.97 | 0.90 | 0.60 | 0.59 | 0.34 |

→ Pro-caspase-6

| 1 | 0.87 | 0.70 | 0.60 | 0.64 | 0.57 |

→ Pro-caspase -7

| 1 | 0.87 | 0.85 | 0.75 | 0.72 | 0.59 |

→ Pro-caspase -9

→ β-actin

0   1   2.5   5   7.5   10 μM WIN-55,212-2

[E]

| 1 | 0.95 | 0.87 | 0.81 | 0.51 | 0.19 |

→ PARP (116KD)

| 1 | 1.02 | 1.30 | 1.69 | 3.10 | 4.41 |

→ PARP (85KD)

→ β-actin

0   1   2.5   5   7.5   10   μM WIN-55,212-2

FIGURE 7. **Effect of WIN-55,212-2 treatment.** *A*, protein expression of p53, Bax, Bcl-2, and AIF; *B*, Bax/BCl-2 ratio; *C*, protein expression of pro-caspase-3 and cleaved caspase-3. *D*, protein expression of pro-caspase 6, 7, and 9. *D* and *E*, cleavage of PARP. As detailed under "Experimental Procedures," the cells were treated with Me₂SO alone or specified concentrations of WIN-55,212-2, and total cell lysates were prepared for immunoblot analysis. The values *above* the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results. The data obtained from the immunoblot analyses of Bax and Bcl-2 were used to evaluate the effect of WIN-55,212-2 on the Bax/Bcl-2 ratio. The densitometric analysis of Bax and Bcl-2 bands was performed using UN-SCAN-IT software, and the data (relative density normalized β-actin) were plotted as Bax/Bcl-2 ratio. Detection of cleaved caspase-3 by confocal fluorescence microscopy; cells were treated with WIN-55,212-2 5.0, 7.5, and 10 μM for 24 h and were stained with antibody Alexa Fluor 488 conjugate.

small proteins known as ckis. Ckis includes the p21/WAF1 and p27/KIP1 protein members. Hence, we studied the modulation in cell cycle regulatory events operational in the G₀–G₁ phase as a mechanism of WIN-55,212-2-mediated cell cycle dysregulation and apoptosis in human prostate cancer cells. It is reported that ckis inhibits the kinase activities associated with cdk-cyclin complexes, thereby modulating the phosphorylation events that play an important role in progression of the cell cycle (30–

34). Recent studies have shown that cell cycle progression through G₀–G₁ phase and apoptosis is regulated by p27/KIP1 (23). We observed a significant induction of p27/KIP1 using immunoblot analysis in WIN-55,212-2-treated cells (Fig. 3*A*). These data suggest that cell cycle dysregulation in androgen-responsive LNCaP cells by WIN-55,212-2 treatment is regulated by ckis involved in G₀–G₁ phase. The progression of cell cycle is modulated via irreversible transition induced by cdks
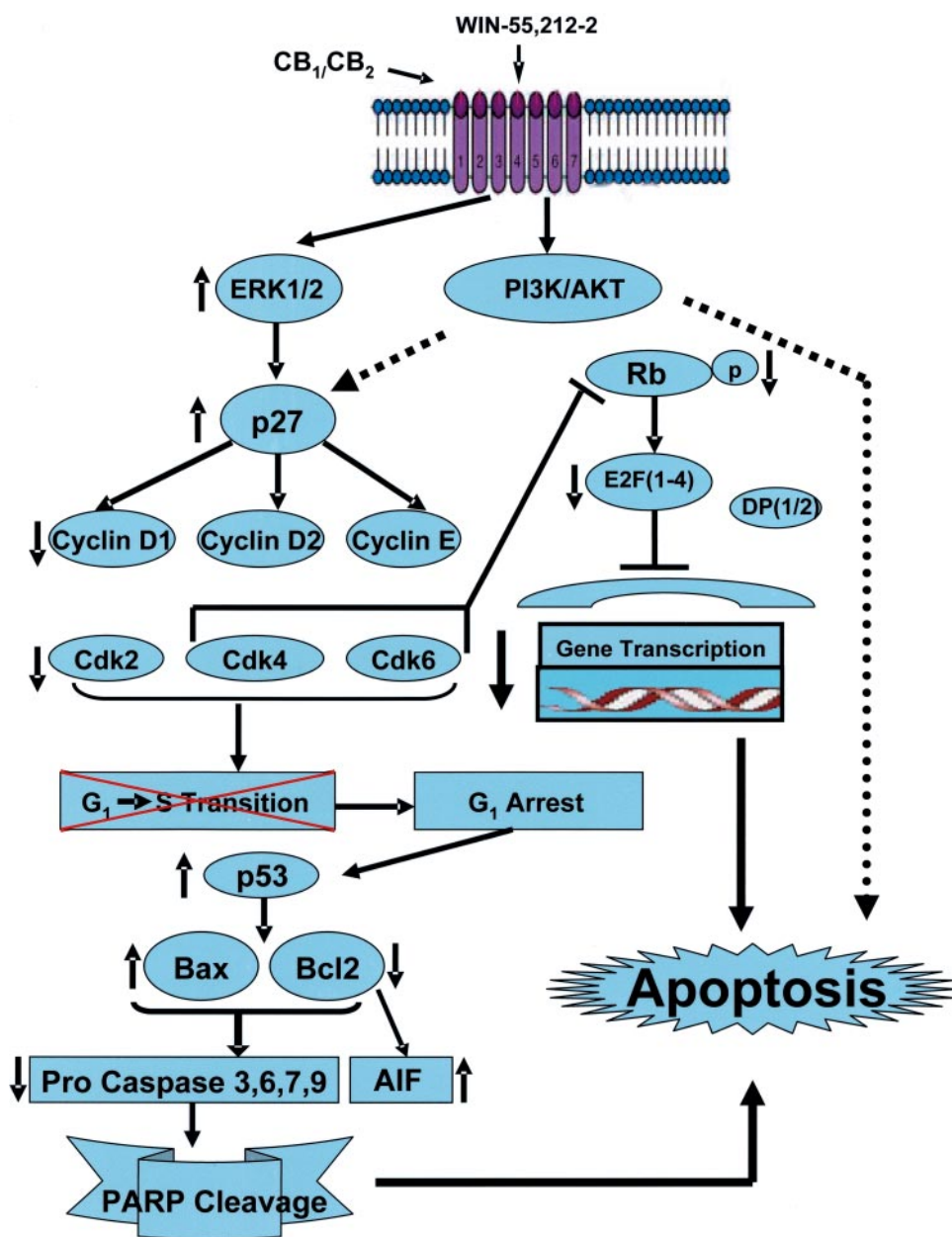
FIGURE 8. **Proposed schematic model for WIN-55,212-2-mediated cell cycle dysregulation and induction of apoptosis.**

E2F target genes through the phosphorylation of pocket proteins by cdks (37, 38, 34). Studies have established that members of retinoblastoma family are capable of exerting growth suppressive activity because of their interaction with E2F/DP heterodimers, which function to trigger the transcription of genes required for cell cycle progression (39, 37). In the present study we investigated the protein levels and the phosphorylation pattern of pRb during WIN-55,212-2-mediated cell cycle arrest and apoptosis. The immunoblot analysis demonstrated a dose-dependent decrease in the pRb and E2F (1–4) family (Fig. 4*A*) and its heterodimers DP-1 and DP-2 (Fig. 4*A*). pRb is largely found in hypophosphorylated form in the early G_1 phase. The hypophosphorylated pRb is able to bind to a subset of E2F/DP heterodimers, thereby inhibiting their transcriptional activation potential (40–43). Taken together, our data demonstrate the involvement of the pRb-E2F/DP pathway during WIN-55,212-2-mediated cell cycle arrest and apoptosis. We observed similar trend when the androgen-insensitive PC3 cell was treated with WIN-55,212-2 (Fig. 4*B*). Our findings demonstrate that treatment of human prostate cancer cell LNCaP with WIN-55,212-2 increases the protein expression of ERK1/2 and inhibits PI3K/AKT at higher doses of WIN-55,212-2 (Fig. 5*A*). The PI3K/AKT signaling pathway is a common response of cells to growth factor stimulation and is essential for survival. ERK1/2 has a dual behavior and is involved in cell proliferation as well as cell cycle arrest. ERK1/2 activation and cell death/proliferation is complex and depends on many factors, one of which is duration of stimulus. Interestingly we found that a sustained increase in ERK1/2 expression at higher doses of WIN-55,212-2 leads to cell cycle arrest and apoptosis. There was no change in the cell morphology when ERK1/2 was inhibited compared with 7.5 μM WIN-55,212-2 (Fig. 6*A*); similarly the ERK1/2 inhibitor significantly reversed the distribution of cells in G_1 phase of the cell cycle (Fig. 6*B*) and also decreased the percentage of apoptotic cells when compared with WIN-55,212-2 treatment alone. The ERK1/2 inhibitor also reversed effects of WIN-55,212-2 on p27/KIP1 and cyclin D1 proteins operative in the G_1 phase of the cell cycle and Bcl-2, an important pro-apoptotic protein (Fig. 6*D*). Similar results

and cyclins (35, 36). We next assessed the effect of WIN-55,212-2 treatment on the cyclins and cdks functional in the G_1 phase of the cell cycle, particularly, cyclins D1, D2, and E (Fig. 3*A*) and cdk2, cdk4, and cdk6 (Fig. 3*A*). WIN-55,212-2 treatment of the cells was found to result in significant reductions of all of these regulatory molecules. Cdk4, Cdk6, and cyclin D1 are involved in early G_1 phase and transition from G_1 to S is regulated by cdk2/cyclin E (36, 34). We observed similar results when androgen-insensitive PC3 cells were treated with WIN-55,212-2 (Fig. 3*B*).

It has been reported that down-regulation of cdk4/6 leads to phosphorylation and inactivation of pRb which then down-regulates with E2F family allowing inhibition of transcription of genes required for S phase (25). The progression of S phase in the cell cycle is accompanied by the transcriptional activation of

were observed when ERK1/2 was silenced using small interfering RNA (Fig. 6*E*).

Members of the Bcl-2 family of proteins are critical regulators of the apoptotic pathway (44, 45) and they can be triggered by up-regulation of p53 protein. Bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis (46). Bcl-2 is found at high levels in more than half of all human tumors and has shown to form a heterodimer complex with the pro-apoptotic member Bax, thereby neutralizing its proapoptotic effects. Therefore, alterations in the levels of Bax and Bcl-2 with shift in the ratio of Bax/Bcl-2 is considered to be a decisive factor in determining whether cells will undergo apoptosis under experimental conditions that promote cell death. In our study, a decrease in Bcl-2 protein expression was observed in LNCaP cells following WIN-55,212-2 treatment (Fig. 7*A*). Importantly, the protein expression of Bax was found to be up-regulated in these cells after 24 h of treatment (Fig. 7*A*). Therefore, the ratio of Bax to Bcl-2 observed in WIN-55,212-2-treated LNCaP cells favored apoptosis (Fig. 7*B*). Our results thus suggest that up-regulation of AIF and Bax and down-modulation of Bcl-2 may be another molecular mechanism through which WIN-55,212-2 induces apoptosis.

Caspases are cysteine proteases, which are formed constitutively in the cells and are normally present as inactive proenzymes. Caspases are activated during apoptosis in a self-amplifying cascade (47). Activation of upstream or initiator caspases, such as caspases 8, 9, and 10, by proapoptotic signals leads to the proteolytic activation of downstream or effector caspases 3, 6, and 7. The effector caspases cleave a set of vital proteins and, thus, initiate and execute the apoptotic degradation of the cell with the typical morphological and biochemical features. Two major pathways of caspase cascade activation have been characterized. One is initiated by ligation of death receptors and the activation of caspase 8. In the other pathway, cytochrome *c* is released from mitochondria in response to a variety of apoptotic stimuli. In the cytosol cytochrome *c* can bind to apaf-1 and, in the presence of dATP or ATP, activates caspase 9 (48, 47). WIN-55,212-2 treatment of cells was found to promote the activation of caspase 9 that activates caspases 3 and 6 in a dose-dependent manner. Using confocal fluorescence microscopy, cells were visualized to cleaved caspase-3 staining at varying doses (Fig. 7*C*). We observed that WIN-55,212-2 treatment caused activation of caspases 9 and 3 with concomitant cleavage of 116-kDa PARP to the 85-kDa product (Fig. 7*E*).

Based on the outcome of this study and the available literature, and as shown in the composite scheme in Fig. 8, we suggest that cannabinoid receptor agonist WIN-55,212-2 induces sustained and prolong activation of ERK1/2, which leads to induction of cyclin kinase inhibitor p27/KIP1, in turn inhibiting cell cycle regulatory molecules resulting in G₁ arrest and apoptosis. Down-regulation of cdk4/6 inhibits pRb, which inhibits protein expression of E2F family of proteins and its heterodimeric partners DP1 and DP2, leading to gene transcription and apoptosis. Because Bax and Bcl-2 play a critical role in induction of apoptosis, alteration of the Bax/Bcl-2 ratio activates caspase signaling, resulting in apoptotic cell death. Hence, we conclude that cannabinoid receptor agonist should be considered as an effective agent for the treatment of prostate cancer. If our hypothesis is supported by *in vivo* experiments, the long term implications of our study could be to develop nonhabit-forming cannabinoid agonist (s) for the management of prostate cancer.

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Appendix 3

96th Annual Meeting
April 16-20, 2005
Anaheim/Orange County, CA

Abstract Number: 2566

Presentation Title: Cannabinoid receptors: a novel target for the
                                   treatment of prostate cancer

Presentation Start/End Time: Monday, Apr 18, 2005, 10:10 AM -10:25 AM

Author Block: *Sami Sarfaraz*, *Farrukh Afaq*, *Vaqar M. Adhami*,
                                   Arshi Malik, *Imtiaz A. Siddiqui*, *Hasan Mukhtar*.
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Because prostate cancer is the most common diagnosed invasive malignancy and second leading cause of cancer related deaths in US male population, developing novel targets and mechanism based agents for its treatment has become a challenging issue. In recent years cannabinoids, the active components of *Cannabis sativa linnaeus* (marijuana) and their derivatives are receiving renewed attention because of their diverse pharmacological activities such as cell growth inhibition, anti-inflammatory effects and tumor regression. Further interest in cannabinoid research came from the discovery of specific cannabinoid system and the cloning of specific cannabinoid receptors. These diversified effects of cannabinoids are now known to be mediated by the activation of specific G protein-coupled "central" CB_1 and "peripheral" CB_2 receptors that are normally bound by a family of endogenous ligands, the endocannabinoids. Here, we show that expression levels of both cannabinoid receptors CB_1 and CB_2 are significantly higher in CA-HPV-10 (virally transformed cells, derived from adenocarcinoma of human prostate tissue), and other human prostate cancer cells LNCaP, DU145, PC3, and CWR22Rν1 than in normal human prostate epithelial (PrEC) and PZ-HPV-7 (virally transformed cells, derived from normal human prostate tissue) cells. Extending this observation we found that WIN-55,212-2 (a mixed CB_1/CB_2 agonist) treatment to androgen-responsive LNCaP cells resulted in (i) dose (1-10 μ M) and time-dependent (24-72 h) inhibition of cell growth, (ii) induction of apoptosis, (iii) decrease in protein and mRNA expression of androgen receptor, (iv) decrease in intracellular protein and mRNA expression of prostate specific antigen (PSA), (v) decrease in secreted PSA levels, and (vi) decrease in protein expression of proliferation cell nuclear antigen and vascular endothelial growth factor. Cells pretreated with specific CB_1 receptor antagonist SR141716 were found to overcome WIN-55,212-2-induced inhibition in cell viability and apoptosis further suggesting a role of CB_1 receptor in WIN-55,212-2 induced cell death . To establish the relevance of these *in vitro* findings to *in vivo* situation, athymic nude mice (6-8 weeks old) were implanted with androgen responsive CWR22Rν1 cells which are known to secrete PSA in the blood stream of the host. As compared to controls mice treated with WIN-55,212-2 (0.5 mg/kg b.wt, i.p, alternate day) resulted in a significant inhibition in the growth of implanted tumors with significant reduction in PSA secretion in serum. In control animals, targeted tumor volume of 1200 mm^3 was reached at 28 days post-tumor inoculation; whereas this tumor volume was attained in 45 days in animals treated with WIN-55,212-2. Our results suggest that WIN-55,212-2 or other non-habit forming cannabinoid receptor agonists could be developed as novel therapeutic agents for the treatment of prostate cancer.

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Appendix 4

2007 AACR Annual Meeting
April 14-18, 2007
Los Angeles, CA

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Abstract Number:	2195
Presentation Title:	**Cannabinoid receptors agonist WIN-55,212-2 inhibits angiogenesis, metastasis and tumor growth of androgen-sensitive prostate cancer cell CWR22Rν1 xenograft in athymic nude mice**
Presentation Start/End Time:	Monday, Apr 16, 2007, 8:00 AM -12:00 PM
Location:	Exhibit Hall, Los Angeles Convention Center
Poster Section:	22
Poster Board Number:	23
Author Block:	*Sami Sarfaraz, Farrukh Afaq, Vaqar M. Adhami, Arshi Malik, Imtiaz A. Siddiqui, Hasan Mukhtar*. Univ. of Wisconsin, Madison, WI

Cannabinoids and their receptors agonists are drawing renewed attention as potential anti-tumor agents. Recently, we have shown that expression levels of both cannabinoid receptors CB_1 and CB_2 are higher in human prostate cancer cells than in normal prostate epithelial cells (*Cancer Res. 65:1635-41, 2005*) and observed that sustained activation of ERK1/2 by cannabinoid receptors agonist WIN-55,212-2 (WIN) leads to G_1 cell cycle arrest and apoptosis in LNCaP cells (*J. Biol. Chem., PMID: 17068343*). To establish *in vivo* relevance of these *in vitro* findings, we implanted athymic nude mice with androgen-responsive CWR22Rν1 cells which form rapid tumors and secrete PSA in the blood stream of the host. As compared to untreated animals, WIN treated mice (0.5 mg/kg b.wt, i.p, alternate day) exhibited significant inhibition in the tumor growth with significant reduction in PSA secretion in the serum. In animals without WIN treatment, targeted tumor volume of 1200 mm^3 was reached at 35 days post-tumor inoculation; whereas this tumor volume was attained in 51 days in WIN treated mice. Since angiogenesis is an essential component to primary tumor growth and metastasis, we next assessed the effect of WIN treatment on the markers of cell proliferation, angiogenesis and metastasis. Protein expression of PCNA, a marker of cell proliferation was considerably lower (45%) in tumors of WIN treated mice as compared to untreated animals. Protein expression of angiopoetins and VEGF, members of the vascular endothelial growth factor family that participate in the formation of blood vessels were also evaluated. Tumor tissues from WIN treated mice had notably lower expression of both angiopoetin-1 (41%) and angiopoetin-2 (38%) and showed marked decrease (47%) in the expression of VEGF positive cells. Loss of function of E-cadherin is associated with progression of cancer by increasing proliferation, invasion, and/or metastasis. We observed that in WIN treated mice E-cadherin expression was 2.5 fold higher as compared to untreated animals. We also found a decrease in the protein expression of cadherin associated proteins β-catenin and α-catenin in tumors of mice treated with WIN. In the next series of experiments we determined the effect of WIN on the expression of proteins involved in metastasis. The balance between matrix metalloproteinases (MMP) and their tissue

inhibitors (TIMP) is an essential factor in the aggressiveness of several cancers. We observed that MMP to TIMP ratio in WIN treated mice was tilted towards TIMP expression suggesting inhibition of MMP expression. Here, we provide *in vivo* evidence for potential use of cannabinoid receptors agonist for slowing tumor growth of androgen sensitive cells in a xenograft model.

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Appendix 5

Cannabinoids for Cancer Treatment: Progress and Promise

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Abstract

Cannabinoids are a class of pharmacologic compounds that offer potential applications as antitumor drugs, based on the ability of some members of this class to limit inflammation, cell proliferation, and cell survival. In particular, emerging evidence suggests that agonists of cannabinoid receptors expressed by tumor cells may offer a novel strategy to treat cancer. Here, we review recent work that raises interest in the development and exploration of potent, nontoxic, and nonhabit forming cannabinoids for cancer therapy. [Cancer Res 2008;68(2):339–42]

Cannabinoid Receptors: A Brief Overview

Cannabinoid refers to a group of chemicals naturally found in the marijuana plant *Cannabis sativa L.* and includes compounds that are either structurally or pharmacologically similar to $\Delta(9)$-tetrahydrocannabinol or those that bind to the cannabinoid receptors. It was earlier thought that cannabinoids exert their physiologic and behavioral effects via nonspecific interaction with cell membranes. Although anticancer effects of cannabinoids were shown as early as 1975 in Lewis lung carcinoma (ref. 1 and references therein), renewed interest was generated little after the discovery of the cannabinoid system and cloning of the specific cannabinoid receptors (1). The diversified effects of cannabinoids are now known to be mediated through the activation of G-protein–coupled receptors that are normally bound by a family of endogenous ligands, the endocannabinoids (1). Two cannabinoid receptors have been characterized and cloned from mammalian tissues: the "central" CB_1 receptor and the "peripheral" CB_2 receptor. CB_1 receptors are found primarily in the brain, specifically in the basal ganglia and in the limbic system, including the hippocampus. They are also found in the cerebellum and in both male and female reproductive systems. CB_2 receptors are almost exclusively found in the immune system, with the greatest density in the spleen (1).

Classification of Cannabinoids

There are three types of cannabinoids. Plant-derived cannabinoids such as $\Delta(9)$-tetrahydrocannabinol and cannabidiol occur uniquely in the cannabis plant; endogenous cannabinoids also known as endocannabinoids such as anandamide and 2-arachidonoylglycerol are produced in the bodies of humans and animals; and synthetic cannabinoids, such as WIN-55, 212-2, JWH-133, and (*R*)-methanandamide (MET), which are developed in a laboratory, bear structural similarities to either natural or the endogenous cannabinoids.

Cannabinoids in the Treatment of Cancer: Progress

Cancer is a disease characterized by uncontrolled division of cells and their ability to spread. This unregulated growth is caused by damage to DNA, resulting in mutations, defects in cell cycle, and apoptotic machinery. Thus, agents that can modulate apoptosis to maintain steady-state cell population by affecting one or more signaling intermediates leading to induction of apoptosis can be useful for targeted therapy of cancer. Hence, there is a need to develop novel targets and mechanism-based agents for the management of cancer. A significant advancement in cannabinoid use in cancer treatment came through the discovery of a potential utility of these compounds for targeting and killing tumors. In the early 1970s, cannabinoids were shown to inhibit tumor growth and prolong the life of mice bearing Lewis lung adenocarcinoma (ref. 1 and references therein). In subsequent studies, molecular mechanisms for these effects were analyzed, and it was found that cannabinoids inhibited tumor cell growth and induced apoptosis by modulating different cell signaling pathways in gliomas and lymphomas, prostate, breast, lung, skin, and pancreatic cancer cells (2–7). Encouraging data about the inhibition of tumor growth by cannabinoid receptor agonists in some animal tumor models, such as wistar rats inoculated with C6 gliomas (ref. 1 and references therein) and xenografts in athymic nude mice implanted with KiMol or MBA-MD-231 breast cancer cells, are beginning to emerge (5). The diversified effects of cannabinoids in modulating cell signaling pathways are depicted in Fig. 1. One puzzling caveat is that in contrast to the tumor-killing properties, some cannabinoids at low doses have also been shown to stimulate the growth of cancer cells *in vitro* (ref. 8 and references therein). Although the observed effects of cannabinoids are complex and sometimes contradictory, there is overwhelming evidence to suggest that cannabinoids can be explored as chemotherapeutic agents for the treatment of cancer (2–7).

Cannabinoids and Gliomas

Glioblastoma multiforme is one of the most dreadful forms of cancer and the most frequent class of malignant primary brain tumors. Antitumor action of two cannabinoid receptor agonists, $\Delta(9)$-tetrahydrocannabinol and WIN-55,212-2 (a mixed CB_1/CB_2 agonist), was shown to be mediated through accumulation of ceramide, which resulted in sustained activation of extracellular signal-regulated kinase (ERK1/2; ref. 2 and references therein). It was also reported that cannabinoids down-regulated phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), and ERK signaling pathways, and activated proapoptotic function of Bad protein, leading to the induction of apoptosis (9). It has also been shown that that selective activation of the CB_2 receptor by JWH-133 mediates apoptosis in glioma cells via enhanced ceramide synthesis *de novo* (ref. 4 and references therein). In addition, a role for stress-regulated protein p8 (also designated as candidate of metastasis-1) and its downstream targets such as activating transcription factor-4 (ATF-4), CAAT/enhancer binding protein homologous protein,
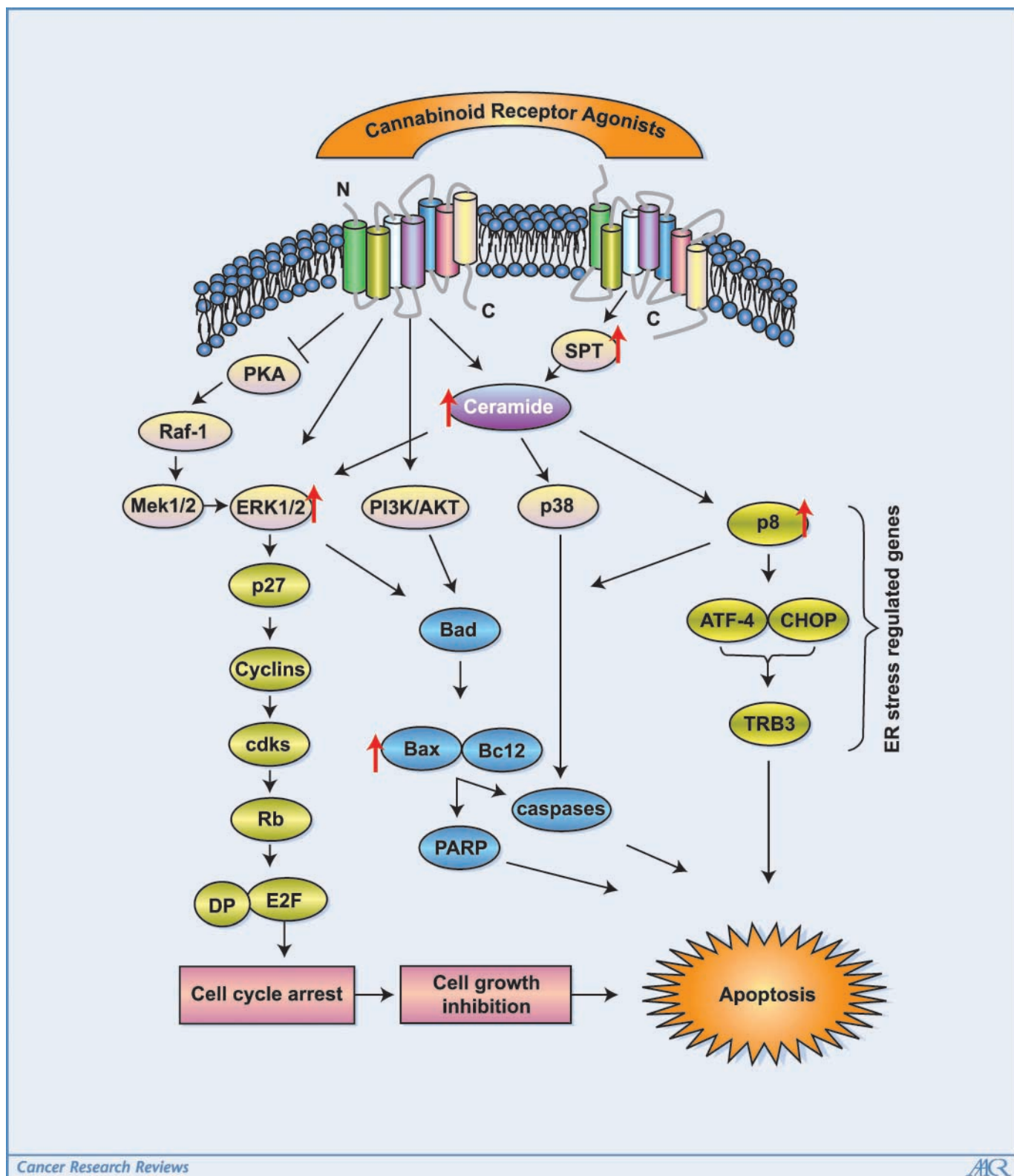
Figure 1. Schematic representation of signaling pathways associated with cannabinoid receptor activation induced by its agonists. Upon receptor binding, cannabinoid receptor agonists inhibit cell proliferation through inhibition of cAMP-dependent protein kinase, which activates mitogen-activated protein kinases (MAPK). Stimulation of ceramide synthesis *via* activation of serine pamitoyltranferase (*SPT*) up-regulates p8, leading to the subsequent induction of apoptosis. Cannabinoid receptor agonists also activates MAPKs and PI3K/Akt pathways; sustained activation of ERK1/2 leads to the induction of cyclin kinase inhibitor p27/KIP1with modulation of cell cycle regulatory molecules, resulting in G_1 arrest and apoptosis. The proposed mechanisms are based on the available literature and are cell specific, and not all pathways are triggered simultaneously. Further studies are needed to unravel the detailed mechanism of action of cannabinoid receptor activation by their agonists. *CHOP,* CAAT/enhancer binding protein homologous protein; *PARP,* poly(ADP)ribose polymerase; *cdk,* cyclin-dependent kinase. *PKA,* cAMP-dependent kinase.

and cell death–inducible kinase (TRB3) was shown as a mechanism of the antitumor action of cannabinoids (2). A phase I clinical trial in nine patients with recurrent glioblastoma multiforme reported a fair safety profile of Δ(9)-tetrahydrocannabinol together with antiproliferative action on tumor cells (10). Contrary to these findings, Massi et al. (11) showed that cannabidiol treatment induces apoptosis in glioma cells *in vitro* and tumor regression *in vivo* through activation of caspases and reactive oxygen species via receptor-independent manner. Although there are few contradictory studies on the mechanism of action of cannabinoids, they all underline the importance of cannabinoids for the treatment of cancer. Hence, further studies are needed to elucidate the mechanism of action of cannabinoids in cancer treatment.

Cannabinoids and Prostate Cancer

The presence of cannabinoid receptors was shown in the prostate tissue and in prostate cancer PC-3 cells. However, it was shown that treatment of PC-3 cells with Δ(9)-tetrahydrocannabinol induced apoptosis via a receptor-independent manner (12). Interestingly, another study from the same group reported that activation of cannabinoid receptors in PC-3 cells stimulated the PI3K/Akt pathway with sequential involvement of Raf-1/ERK1/2 and nerve growth factor induction (ref. 13 and references therein). We have recently shown that the expression levels of both cannabinoid receptors CB_1 and CB_2 are significantly higher in human prostate cancer cells compared with normal prostate epithelial cells. Based on this observation, LNCaP cells were treated with WIN-55,212-2, which resulted in inhibition of cell growth and induction of apoptosis (ref. 4 and references therein) with an arrest of the cells in the G_0-G_1 phase of the cell cycle. This WIN-55,212-2–induced cell cycle arrest was associated with a sustained activation of ERK1/2 (4). To establish *in vivo* relevance of our *in vitro* findings, we showed that in CWR22Rν1 xenograft model, WIN-55,212–treated mice exhibited significant inhibition in the tumor growth with remarkable reduction of prostate-specific antigen secretion in the serum (14). Nithipatikom et al. (13) showed that increasing endogenous 2-arachidonoylgl and its stable analogue noladin ether inhibited invasion of androgen-independent prostate cancer PC-3 and DU-145 cells. Antiproliferative and apoptotic effects of endogenous cannabinoids anandamide in human prostate cancer cell lines LNCaP, DU145, and PC3 were found to be mediated through down-regulation of epidermal growth factor receptor (EGFR) and accumulation of ceramide (15). Interestingly, anandamide analogue (R)-methanandamide was shown to have a mitogenic effect on LNCaP cells at very low doses (16).

Cannabinoids and Breast Cancer

It has been shown that anandamide, potently and selectively, inhibited proliferation of human breast cancer cells. This antiproliferative activity of anandamide was accompanied by a reduction of cells in the S phase of the cell cycle and suppression of prolactin receptor (ref. 5 and references therein). Ligresti, Moriello, and colleagues (5) have shown antitumor activities of five natural cannabinoids, cannabidiol, cannabigerol, cannabichromene, cannabidiol acid, and Δ(9)-tetrahydrocannabinol, and suggested that cannabidiol was the most potent inhibitor of breast cancer cell growth. Both cannabidiol and the cannabidiol-rich extract also inhibited the growth of MDA-MB-231 breast carcinoma cells in athymic nude mice. In another study, (R)-methanandamide reduced the number and size of metastatic nodes, and this effect

was reversed by CB_1 receptor antagonist SR141716A. (R)-methanandamide–treated cells also showed decreased phosphorylation of focal adhesion–associated protein kinase and Src, and tyrosine kinases involved in migration and adhesion, suggesting that CB_1 receptor activation might represent a novel therapeutic strategy to slow down the growth of breast carcinoma and to inhibit its metastatic diffusion *in vivo* (17). Contrary to these findings, McKallip et al. (18) have earlier shown that Δ(9)-tetrahydrocannabinol enhanced breast cancer growth and metastasis specifically in cells expressing low levels of cannabinoid receptors by suppressing the antitumor immune response, suggesting that cannabinoid exposure may increase the incidence of breast cancer as well as other cancers that do not express cannabinoid receptors.

Cannabinoid and Lung Cancer

Lung cancer survival figures argue powerfully for new approaches to control this disease by agents that could reverse, suppress, or completely halt tumor development. Guzman (ref. 1 and references therein) reported for the first time that Lewis lung adenocarcinoma growth was retarded by the p.o. administration of Δ(9)-tetrahydrocannabinol, and based *in vitro* studies, inhibition of DNA synthesis was identified as a mechanism for these effects. Another study showed that concentrations of Δ(9)-tetrahydrocannabinol comparable with those detected in the serum of patients after Δ(9)-tetrahydrocannabinol administration accelerate proliferation of lung cancer cells (ref. 8 and references therein). Treatment of lung carcinoma cell line NCI-H292 with nanomolar concentrations of Δ(9)-tetrahydrocannabinol led to accelerated cell proliferation that was dependent on EGFR-mediated activation of ERK1/2 as well as PKB/Akt signaling (ref. 8 and references therein). Recently, it has been shown that Δ(9)-tetrahydrocannabinol treatment inhibited epidermal growth factor–induced phosphorylation of ERK1/2, c-Jun-NH$_2$-kinase1/2, and Akt in A549 human lung cancer cell line as well as suppression of metastasis and s.c. tumor growth in severe combined immunodeficient mice (8).

Cannabinoid and Skin Cancer

Melanoma is responsible for the greatest number of skin cancer–related deaths worldwide. It was reported that CB_1 and the CB_2 receptors are expressed in normal skin and skin tumors of mice and humans. *In vitro* studies showed that activation of cannabinoid receptors induced the apoptotic death of tumorigenic epidermal cells, without affecting the nontransformed epidermal cells. Administration of WIN-55,212-2 or the selective CB_2 agonist JWH-133 was shown to result in growth inhibition of malignant tumors in nude mice (ref. 6 and references therein). Another study showed that activation of these receptors decreased tumor growth, angiogenesis and metastasis of melanomas in mice, and inhibited proliferation via inhibition of Akt pathway and hypophosphorylation of retinoblastoma in melanoma cells (6). These two studies offer an exciting opportunity to further explore the use of cannabinoids for the treatment and management of melanoma.

Cannabinoid and Pancreatic Cancer

Pancreatic cancer ranks as one of the most fatal forms of cancer, and therefore, new strategies aimed at improving the prognosis of this deadly disease are warranted. Recently, it was shown that cannabinoid administration leads to apoptosis of pancreatic tumor cells via CB_2 receptor and ceramide-dependent up-regulation of p8

and ATF-4 and TRB3 stress–related genes (7). Another study showed that CB_1 receptor antagonist AM251–induced cell death in pancreatic MIAPaCa-2 cells occurred via receptor-independent manner (19). Although the two studies describe contrasting mechanism of action of cannabinoids, both underline the importance of cannabinoids for the treatment of pancreatic cancer. In depth studies are therefore warranted to identify the mechanism of action of cell death induced by cannabinoids in pancreatic cancer.

Cannabinoid and Lymphoma

Studies show that exposure of murine lymphoma tumors EL-4, LSA, and P815 to Δ(9)-tetrahydrocannabinol *in vitro* led to a significant reduction in cell viability and an increase in apoptosis, and EL-4 tumor–bearing mice led to a significant reduction in tumor load, increase in tumor-cell apoptosis, and increase in survival of tumor-bearing mice (ref. 20 and references therein). Similar observations were made by Flygare et al. (20) who treated mantle cell lymphoma (MCL) cells with cannabinoid receptor ligands and found a decrease in cell viability, whereas control cells lacking CB_1 were not affected. Recently, Gustafsson et al. (3) reported that cannabinoid receptor–mediated apoptosis induced by (R)-methanandamide and WIN-55,212-2 in MCL was associated with ceramide accumulation and p38. These data suggest that targeting CB_1 and CB_2 receptors by their agonists may have therapeutic potential for the treatment of lymphoma.

Conclusions and Future Prospects: Promise

Cannabinoids, the active components of marijuana and their other natural and synthetic analogues have been reported as useful adjuvants to conventional chemotherapeutic regimens for preventing nausea, vomiting, pain, and for stimulating appetite.

Before the discovery of specific cannabinoid systems and receptors, it was speculated that cannabinoids produced their effects via nonspecific interaction with cell membranes. Cannabinoids are proving to be unique based on their targeted action on cancer cells and their ability to spare normal cells. Variation in the effects of cannabinoids in different cell lines and tumor model could be due to the differential expression of CB_1 and CB_2 receptors. Thus, overexpression of cannabinoid receptors may be effective in killing tumors, whereas low or no expression of these receptors could lead to cell proliferation and metastasis because of the suppression of the antitumor immune response. It is also reported that low doses of cannabinoid administration accelerate proliferation of cancer cells instead of inducing apoptosis and, thereby, contribute to cancer progression. Till date, very little is known about the mechanism of action of cannabinoids. There is need for further in-depth studies to elucidate the precise mechanism of cannabinoid action in cancer cells. Safety of Δ(9)-tetrahydrocannabinol administration has been determined, and a dose escalation regimen showed that cannabinoid delivery was safe and could be achieved without overt psychoactive effects. In view of the fair safety profile of most cannabinoids together with their antiproliferative action on tumor cells, clinical trials are required to determine whether cannabinoids could be used for the inhibition of tumor growth in a clinical setting. If this could be established, then one can hope that nontoxic, nonhabit forming cannabinoids could be developed as novel therapeutic agents for the treatment of cancer.

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